

**Search for Genes Involved in the Synthesis  
of Poly(L-malate) in the Plasmodium of  
*Physarum polycephalum***

Dissertation

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# CONTENTS

Abbreviations.....	7
I Introduction .....	9
1 Physarum polycephalum .....	9
1.1 Taxonomy.....	9
1.2 Life cycle .....	10
1.3 Differential gene expression in amoebae and plasmodia .....	12
2 $\beta$ -poly(L-malic acid) (PMLA).....	14
2.1 Chemical structure and natural sources .....	14
2.2 PMLA in Physarum polycephalum.....	14
2.3 Biosynthesis of PMLA in <i>Physarum polycephalum</i> .....	15
2.3 Biodegradation .....	16
3 Goal of the thesis.....	18
II Materials und methods .....	19
1 Materials .....	19
1.1 Apparatus and accessories .....	19
1.2 Consumable goods.....	20
1.3 Chemicals.....	21
1.4 Enzymes, antibodies and vectors .....	23
1.5 Kits .....	25
1.6 Organisms .....	25
1.7 Standard markers.....	26
1.7.1 DNA standard markers.....	26
1.7.2 Protein standard marker .....	27
1.9 Solutions and media for cell culture.....	28
1.9.1 Solutions and media for plasmodia cultures .....	28
1.9.2 Solutions and media for amoebae cultures .....	29
1.10 Solutions for analysis of nucleic acids .....	32
1.10.1 Solutions for mRNA isolation using Dynabeads <sup>®</sup> oligo(dT) <sub>25</sub> .....	32
1.10.2 Solutions for agarose gel electrophoresis .....	33

1.10.3	Solutions for suppression subtracted hybridization and knock- down assays	34
1.10.4	Solutions and media for transformation of DNA	34
1.11	Solutions for SDS-PAGE and Western Blotting	36
1.11.1	Solutions for cell lysis and SDS electrophoresis	36
1.11.2	Solutions for Western Blotting:	38
1.12	Solutions for quantitative analysis of PMLA	39
2	Methods	40
2.1	Cell culture	40
2.1.1	Cultivation of plasmodia	40
2.1.1.1	Cultivation of microplasmodia	40
2.1.1.2	Induction of Spherules	41
2.1.1.3	Cultivation of macroplasmodia	41
2.1.2	Cultivation of amoebae	41
2.1.2.1	Growth of amoebae on DSDM agar plates	41
2.1.2.2	Preparation of amoebal stock culture	42
2.1.2.3	Growth of amoebae in axenic liquid medium	42
2.2	Isolation of nucleic acids	43
2.2.1	Isolation of total RNA	43
2.2.2	Poly A <sup>+</sup> mRNA isolation from total RNA using Dynabeads <sup>®</sup> Oligo(dT) <sub>25</sub>	44
2.2.2.1	Principle	44
2.2.2.2	Procedures	44
2.2.3	Isolation of DNA using QIAquick PCR Purification Kit	45
2.2.3	Isolation of DNA from agarose gel using QIAquick Gel Extraction Kit	45
2.2.5	Isolation of Plasmid DNA using Nucleospin <sup>®</sup> Plasmid Kit	46
2.2.6	Isolation of plasmid DNA using QIAGEN Plasmid Maxi Kit	47
2.3	Analysis and amplification of nucleic acids	48
2.3.1	Quantification of nucleic acids	48
2.3.2	Polymerase chain reaction (PCR)	49
2.3.3	Real time PCR	50
2.3.3.1	Principle	50

2.3.3.2	Experimental procedure .....	55
2.3.3.2.1	Absolute quantification .....	55
2.3.3.2.2	Relative quantification .....	57
2.3.4	RT-PCR.....	58
2.3.4.2	First-strand cDNA synthesis using oligo(dT) primer .....	59
2.3.4.3	cDNA synthesis using CapFinder .....	60
2.3.4.3.1	Principle .....	60
2.3.4.3.2	Procedure .....	62
2.3.4.4	5' RACE.....	63
2.3.4.4.1	Principle .....	63
2.3.4.4.2	Procedures.....	65
2.4	Cloning of DNA fragments.....	66
2.4.1	Principle .....	66
2.4.2	Procedures .....	68
2.4.2.1	Ligation of DNA fragment with a pGEM <sup>®</sup> -T vector .....	68
2.4.2.2	Transformation of ligated DNA .....	68
2.4.2.3	Isolation of plasmid DNA .....	69
2.4.2.3	Verification of DNA insertion by restriction enzyme digestion.....	69
2.5	Suppression subtractive hybridization .....	70
2.5.1	Principle of suppression subtractive hybridization .....	70
2.5.2	Experimental procedure .....	73
2.5.2.1	Isolation of poly(A) <sup>+</sup> RNA.....	73
2.5.2.2	First-stranded cDNA synthesis .....	73
2.5.2.3	Analysis of synthesized cDNA .....	74
2.5.2.4	Long-distance PCR (LD-PCR).....	75
2.5.2.5	BstUI digestion .....	78
2.5.2.6	Adapter ligation.....	79
2.5.2.7	Analysis of ligation.....	79
2.5.2.8	First hybridization .....	81
2.5.2.9	Second hybridization .....	81
2.5.2.10	Selective PCR Amplification .....	82
2.5.2.11	Nested PCR.....	83
2.5.2.12	PCR analysis of subtraction efficiency.....	84
2.5.2.13	Cloning and analysis of subtracted cDNAs.....	85

2.6	Knock-down assays.....	86
2.6.1	Antisense assays using vector encoded for enhanced yellow fluorescent protein .....	86
2.6.1.1	Principle.....	86
2.6.1.2	Procedures .....	87
2.6.1.2.1	Cell culture and microinjection .....	87
2.6.1.2.2	Fluorimetric measurement .....	89
2.6.1.2.3	Western Blotting.....	89
2.6.2	RNAi assays.....	89
2.6.2.1	Principle.....	89
2.8	Analysis of proteins by SDS-PAGE and Western Blotting .....	92
2.8.1	Preparation of crude extract of macropasmodium .....	92
2.8.2	Determination of protein concentration using the Bradford assay ....	92
2.8.3	SDS-PAGE.....	93
2.8.4	Western Blotting .....	94
2.9	Quantitative analysis of PMLA.....	96
2.9.1	Principle .....	96
2.9.2	Procedures .....	97
III	Results .....	99
1	Suppression subtractive hybridization .....	99
1.1	Analysis of cDNA.....	99
1.2	LD-PCR .....	101
1.3	Analysis of ligation.....	102
1.4	Analysis of subtraction efficiency.....	103
1.5	Analysis of subtracted cDNAs .....	104
2	Knock-down assays of subtracted cDNAs .....	112
2.1	Verification of EYFP expression in the plasmodium .....	112
2.2	Knock-down assays using ds RNA.....	114
2.2.1	Verification of RNAi effect by monitoring phenotypically change....	114
2.2.2	Quantification of RNAi effect by Real-time PCR.....	123
2.2.3	Knock-down analysis by quantification of PMLA level.....	126
3	Quantification of polymalatase at cDNA level .....	127

V	Discussion and outlook .....	130
1	Suppression subtractive hybridization .....	130
2	Knock-down assays of the subtracted cDNAs .....	132
2.1	Verification of EYFP expression in the plasmodium .....	132
2.2	Knock-down assay using dsRNA.....	133
3	Quantification of polymalataase at cDNA level .....	134
4	Outlook .....	135
VI	Conclusion .....	136
VII	References .....	138
VIII	Attachment .....	146
8	cDNA sequences.....	146
	Erklärung.....	172

## Abbreviations

Amp	ampicillin
APS	ammoniumpersulfate
ATP	adenosinetriphosphate
bp	basenpair
BSA	bovine serum albumine
cDNA	complementary DNA
CTAB	Cetyltrimethylammonium bromide
D	Dalton
ddH <sub>2</sub> O	double distilled water
DEPC	diethylpyrocarbonate
DMF	N, N'-dimethylformamid
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide (N=A,T,G,C)
dsRNA	double-strand RNA
DTT	dithiothreitol
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylendiamintetraacetic acid
Em	emission maximum
Ex	excitation maximum
EYFP	enhanced yellow fluorescent protein
g	1. gram, 2. gravitationscoefficient
h	hour
HEPES	N-2-hydroxyethylpiperazin-N'-2-ethansulfic acid
Ig	immunglobulin
IPTG	isopropylthio- $\beta$ -galactoside
kb	kilobasepair
kD	kilodalton
l	liter
LB	Luria-Bertani
M	mol/l
mA	milliampere
mg	milligram
min	minute
ml	milliliter



mM	millimolare
MOPS	3-N-morpholinopropan-sulfonic acid
mRNA	messenger RNA
nm	nanometer
OD	optical density
PAGE	polyacrylamidgelelectrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PMLA	$\beta$ -Poly(L-Malat)
PVDF	polyvinylidenfluoride
RACE	Rapid Amplification of cDNA Ends
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	RNase inhibitor
RT	reverse transkriptase
SDS	sodiumdodecylsulfate
s	second
SSH	suppression subtractive hybridization
ssRNA	single-strand RNA
T	thymidin
Taq	<i>Thermus aquaticus</i>
TE	tris-EDTA
RNAi	RNA Interferenz
TEMED	N, N, N', N' - tetramethylendiamin
Tris	tris(hydroxymethyl) - aminomethan
U	unit
rpm	round per minute
V	volt
X-Gal	5-Bromo-4-Chloro-3-indolyl- $\beta$ -Galactosid

# I Introduction

## 1 *Physarum polycephalum*

### 1.1 Taxonomy

The slime molds were first described in the mid-1800s as one of the earliest eukaryotes. Three distinct groups are defined: cellular (dictyostelid), plasmodial (myxogastrid), and protostelid slime molds. *Physarum polycephalum* belongs to the Myxogastria, the plasmodial or true slime molds. The Myxogastria is classified as following [Aldich et al. 1982; Sitte 1998]:

Phylum: Mycetozoa

Class: Myxogastria

Subclass: Myxogastromycetidae

Order: Physarales

Family: Physaraceae

Genus: *Physarum*

Species: *Physarum polycephalum*

However, the exact phylogenetic position of the Mycetozoa is not clear. Molecular analyses of the elongation factor-1 $\alpha$  encoding genes from one member of each division strongly support the Mycetozoa as a monophyletic group, probably more closely related to the animals and fungi than to plants [Baldauf and Doolittle, 1997].

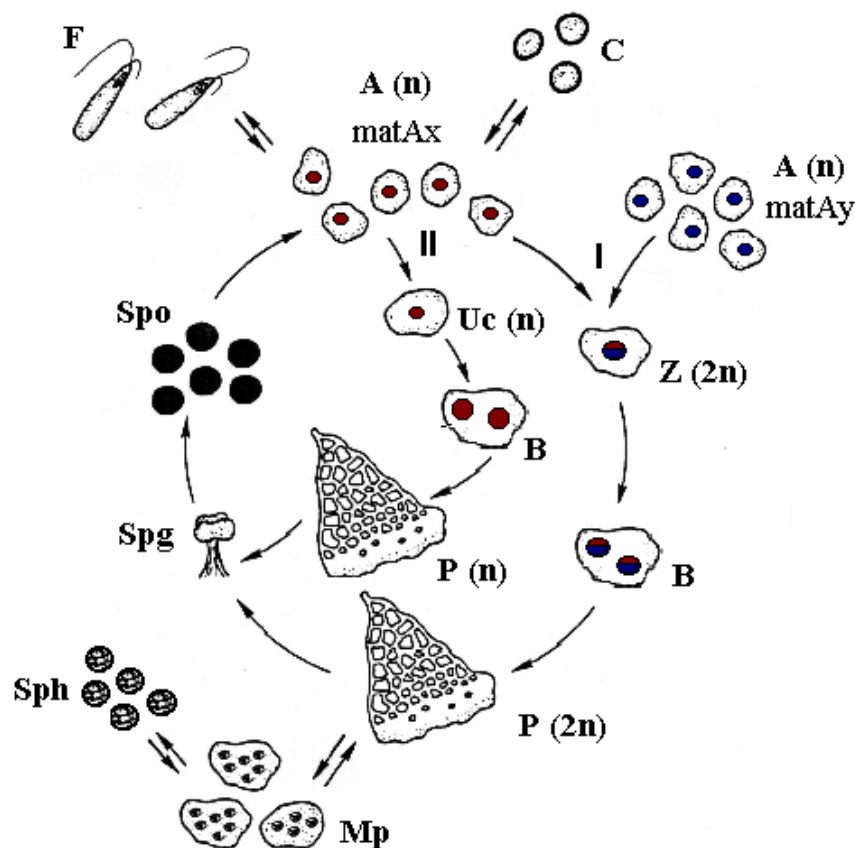
## 1.2 Life cycle

*Physarum polycephalum* is a versatile organism, displaying several alternative cell types and developmental transitions. Uninucleate amoebae and multinucleate plasmodia constitute the two vegetative growth phases in the life cycle.

Amoebae are haploid, uninucleate cells with a diameter of 10 to 20  $\mu\text{m}$ . Amoebae usually feed by phagocytosis on bacteria, fungal spores and other micro-organism. In the laboratory, amoebae are cultured on bacterial lawns, but strains carrying mutant alleles of the *axe* genes are as well capable of growing in liquid axenic medium. In moist conditions, amoebae transform into flagellates, which are unable to undergo mitosis and to feed. The flagellates readily revert to amoebae in dry condition. In adverse condition, such as starvation, amoebae reversibly transform into cysts. In favorable condition they mate and develop into plasmodia. Strains of *P. polycephalum* amoebae can be grouped into two general types - heterothallic and apogamic - depending on their ability to form plasmodia in clones. All samples of *Physarum polycephalum* amoebae, so far isolated from nature show a heterothallic life cycle: diploid plasmodia are formed by the fusion of amoebae of different mating types (Figure. 1, I). The functions of several mating-type loci have been identified. However, only one locus, *matA*, controls the developmental transition from one cell type to the other. Only if fusing amoebae carry different alleles of *matA*, cell fusion is soon followed by nuclear fusion and the zygote develops into a plasmodium. Mutations at *matA* give rise to apogamic amoebal strains in which haploid amoebae are able to differentiate into haploid plasmodia without fusion, a phenomenon also called as “selfing” (Figure1, II). As in sexual development, apogamic amoebae undergo a period of proliferation before clonal plasmodium formation is initiated. It was found that proliferating amoebae secrete a chemical diffusible substance, which is supposed to act as an inducer for plasmodium formation [Youngman et al., 1977]. Soon after the required concentration of the inducer is reached, cells become “committed” to development. A committed uninucleate cell then continues to grow for more than twice the length of a normal amoebal cell cycle. At the end of this extended cell cycle, the uninucleate cell becomes binucleate by mitosis without cytokinesis [Bailey et al., 1987]. From the binucleate stage on, developing cells also

frequently fuse with one another, leading to a rapid increase in size and giving rise to a plasmodium.

The plasmodium of *Physarum polycephalum* is a yellow, multinucleate macroscopic syncytium with an intricate network of veins. Locomotion occurs as a result of protoplasmic streaming of the cell contents within the veins. Plasmodium feeds by phagocytosis on bacteria, amoebae and other micro-organism. In the laboratory, plasmodium can be grown axenically on agar or in liquid culture. In liquid shaken culture, plasmodium fragments into smaller microplasmodia. Grown on agar, the plasmodium can reach a diameter of more than 30 cm. In contrast to the situation in amoebae, plasmodial microtubules do not radiate from an organizing centre during the interphase. Instead, a sparse network is formed in the cytoplasm [Salles-Passador et al., 1991]. The mitotic spindle in plasmodium is nucleated by an intranuclear organizing centre and the nuclear membrane remains intact throughout this “closed” mitosis [Havercraft and Gull, 1983]. The nuclei within a plasmodium undergo mitosis synchronously without cytokinesis. The absence of cytokinesis and fusion between plasmodia lead to a rapid increase in plasmodial size. A set of loci (i.e. *fusA*, *fusB*) were found to affect fusion between plasmodia: genetically identical plasmodia readily fuse with one another. In adverse conditions, plasmodia reversibly transform into dormant sclerotia (spherules), when starved in the light, sporangia are formed. In favourable conditions, spores hatch to release amoebae or flagellates, thus completing the cycle.



**Figure.1:** Life cycle of *Physarum polycephalum*: I = heterothallic cycle, II = apogamic cycle; F = flagellate, A = amoebae, C = cysts, Z = zygote, Uc = uninucleate cell, B = binucleate cell, P = plasmodium, Mp = microplasmodien, Sph = spherules, Spg = sporangium, Spo = spores, matAx and matAy = alleles of mating-type locus, n = haploid, 2n = diploid [according to Anderson and Dee, 1990]

### 1.3 Differential gene expression in amoebae and plasmodia

The differences in cellular organization and behaviour between amoebae and plasmodia are the result of difference in gene expression. Comparison of the proteins present in amoebae and plasmodia by two-dimensional gel electrophoresis indicated that 26% of total examined proteins were cell-type-specific and were found only in amoebae or plasmodia. Among all proteins present in both cell types, 18% showed substantial differences in expression level [Turnock et al., 1981]. In several cases, different members of multigene families are expressed. For example,

amoebae and plasmodia express different myosin, fragmin and profilin genes [Bailey, 1995]. In addition, different members of the tubulin multigene family were found:  $\alpha$ 1-,  $\alpha$ 3, and  $\beta$ 1 tubulin isotypes were detectable in amoebae, while  $\alpha$ 1-,  $\alpha$ 2,  $\beta$ 1 and  $\beta$ 2-tubulin isotypes were observed in plasmodia [Burland et al., 1993]. Table 1 shows an overview of cell-type-specific gene expression.

plasmodial-specific gene products	amoebal-specific gene products
Actin-fragmin kinase	
	ABP
hap-p	
Plasmin C	
Profilin P	Profilin A
	Actin D
$\alpha$ 1 B-tubulin	$\alpha$ 1 A-tubulin
$\alpha$ 2 B-tubulin	
$\beta$ 2-tubulin	
Myosin heavy chain P	Myosin 18K light chain A
Myosin 18K light chain P	Myosin 18K light chain A
Fragmin P	Fragmin A

Table 1 Cell-type-specific gene expression in *P. polycephalum* [according to Bailey, 1995]

## 2 $\beta$ -poly(L-malic acid) (PMLA)

### 2.1 Chemical structure and natural sources

Poly(L-malic acid) (PMLA) is a water soluble and optically inconspicuous polymer, derived from the ester linkage between the hydroxyl group and the carboxyl group of the monomeric L-malic acid units. The polymer was first discovered in the late 1960's as an inhibitor of acidic proteases in *Penicillium cyclopium* [Shimada et al., 1969]. Several years later PMLA was found in the myxomycete *Physarum polycephalum* [Fischer et al., 1989], in the mitosporic fungus *Aureobasidium* sp [Nagata et al., 1993] and in other filamentous fungi [Rathberger et al., 1999]. Among several possible isomers only the unbranched  $\beta$ -poly(L-malic acid) has been found in biological systems.  $\beta$ -poly(L-malic acid) from the mitosporic fungi is of low molecular mass (5-10 kDa) and contains short chains, whereas  $\beta$ -poly(L-malic acid) produced by *Physarum polycephalum* and other myxomycetes has long chains with a high molecular mass between 50 and 300 kDa

### 2.2 PMLA in *Physarum polycephalum*

Of the various cell types in the life cycle of *Physarum polycephalum*, only the plasmodium contains  $\beta$ -poly(L-malate). The polymer is concentrated in the nuclei in an amount comparable with that of DNA and histones. The physical and biochemical properties of the polymer suggest that one of the biological functions of PMLA is to interact with nuclear proteins by mimicking DNA. Such interactions were indeed found by *in vitro* studies showing that PMLA formed tight complexes with histones, DNA polymerase  $\alpha$ /primase complex and other nuclear proteins [Angerer and Holler, 1995]. The activities of the DNA-polymerase- $\alpha$  is inhibited by the complex formation. It is proposed that PMLA binds to the DNA site of the polymerase and competitively inhibits the polymerase activity. The inhibition is reversed by spermidine, histones and biogenic amines, suggesting that PMLA may also serve as a storage place for histones. Histones are synthesized during the S phase, while DNA polymerase  $\alpha$  is

constitutively expressed. It is proposed that the newly synthesized histones displace DNA polymerase  $\alpha$  from poly(L-malate) by competition. The released polymerase is then active in DNA replication until histone synthesis is over. Histones are used in the formation of nucleosomes with newly replicated DNA, while free poly(L-malate) reassociates with DNA polymerase  $\alpha$  at the beginning of the G2 phase and thus terminates DNA synthesizing activity. Competition with periodically synthesized histones or other nucleic proteins may affect on growth and cell cycle. Experiments with injected PMLA into plasmodia increased growth rate and shortened cell cycle duration, supporting this assumption [Karl et al., 2004]. Thus, Poly(L-malate) functions as a storage molecule and a mobile matrix for nuclear proteins, and may involves in molecular events, which are responsible for the synchronization in plasmodium.

## 2.3 Biosynthesis of PMLA in *Physarum polycephalum*

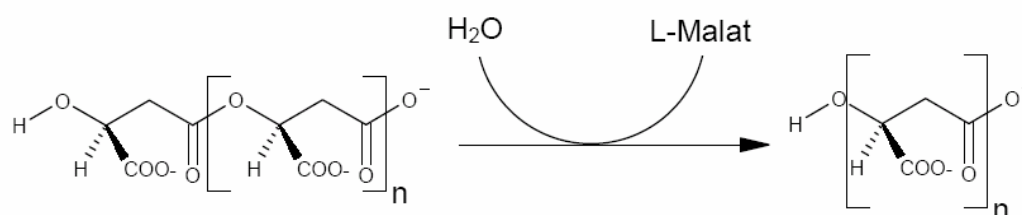
In *Physarum polycephalum*,  $\beta$ -poly(L-malic acid) is synthesized from L-malate derived from D-glucose through the glycolytic pathway and the tricarboxylic acid cycle. The polymerization of PMLA is preceded by chemical activation of L-malate. Two possible types of activated L-malate are discussed:  $\beta$ -L-malyl-AMP and  $\beta$ -L-malyl-CoA. Since no malyl-CoA ligase has been found in eukaryotes and NMR analysis indicated the in vivo synthesis of  $^{13}\text{C}$ -PMLA from D-[1- $^{13}\text{C}$ ] glucose also via pyruvate carboxylation and oxalacetate reduction [Lee et al. 1999], L-malate is probably activated as L-malyl-AMP. To yield malyl-AMP and pyrophosphat, ATP is cleaved between the  $\alpha$ ,  $\beta$  phosphates. Thus, the synthesis of PMLA should be inhibited by  $\alpha$ ,  $\beta$ -noncleavable ATP analogues. It was indeed found that the in vitro synthesis of PMLA was inhibited, when L-[14C]malate was coinjected with Adenosine-5'-( $\alpha$ , $\beta$  -methylene) triphosphate, a noncleavable analogue of ATP. In contrary, the synthesis was not affected by desulfo CoA, a competitive inhibitor of Coenzym A. All these results suggest that L-malate is likely activated as L-malate. Miss occurs, if carbonate is present in the culture medium, malic acid can be directly synthesized by carboxylation of pyruvate and reduction of oxalacetate. The use of tricarboxylic acid cycle as a precursor supplied is no longer necessary.



Due to its spontaneous inactivation during homogenization procedures, attempts to isolate PMLA-synthetase have failed. The inactivation probably involves a cell injury kinase pathway. Since the synthesis of PMLA was blocked by guanosine 5'-( $\beta,\gamma$ -methylene) triphosphate, the injury signal is likely to be GTP dependent [Willibald et al., 1999].

## 2.3 Biodegradation

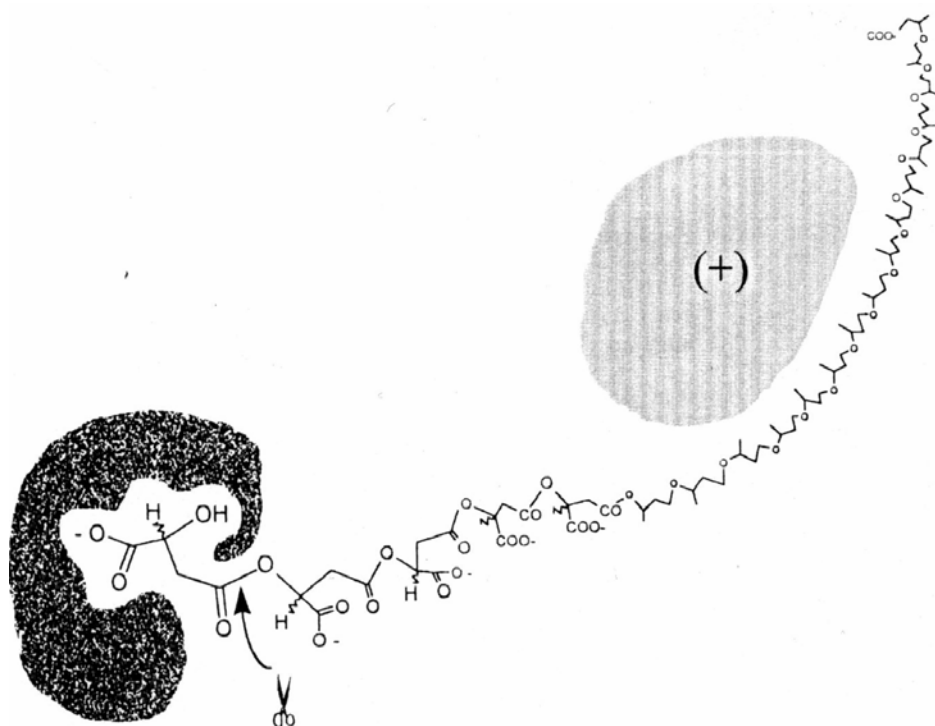
In highly PMLA producing strains, the polymer is released from the nuclei into cytoplasm and finally into the culture medium, where it is degraded to L-malate by a specific hydrolase (Figure. 2). The enzyme, also termed as polymalatase, has been purified and characterized from both the plasmodia and culture medium. The preparation contained several polypeptides, which could not be separated without losing the hydrolase activity. Glycosylated polypeptides of 68 kDa and 97 kDa were identified as polymalatase, as the intensities of only these two bands correlated with hydrolase activity. Results of a two-dimensional gel electrophoresis with non-denaturing gradient gel in the first dimension suggested that both proteins were derived from a 200-kDa precursor, probably by proteolytic fragmentation.



**Figure 2** Hydrolysis of polymalate

Experiments showed an accumulation of smaller oligomers (11-mer and 12-mer), indicating a processive depolymerization. Structure-function experiments also indicated that the hydrolase contains two binding sites. The OH-terminus of the substrate is anchored by specific binding of the penultimate malyl residue. The hydrolase cleaves the adjacent ester bond and liberates L-malate while moving downstream from this catalytic site. The second binding site is responsible for the

processive hydrolysis and is functioning by electrostatic interactions with the polymer chain (Figure 3). Inhibition studies showed no effect of metal ions and serine protease inhibitors, suggesting polymalatase is neither a metallo nor a serine esterase [Korher et al., 1995]. In contrary, the enzyme was completely inactivated by *p*-mercuribenzoate, *N*-bromosuccinimide and D-gluconolactone. In this regard the hydrolase showed similarities to  $\beta$ -glucosidase from *Physarum polycephalum* [Morita et al., 1993], 1,3- $\beta$ -glucanase from *Penicillium oxalicum* [Copa-Patino et al., 1989] and  $\beta$ -D-glucanase from *Candida utilis* [Notario et al., 1976].



**Figure.3** Schematic representation describing the substrate binding of polymalatase [Gasslmaier et al., 2000]

Maximum catalytic rates of polymalatase were measured at pH 3.5 and were remarkably decreased with increasing pH. According to the pH optimum, the intracellular pH (about 6.5 in the cytosol) should be highly unfavourable for catalysis. Indeed, the hydrolase in cytoplasmic preparation was only marginally active, suggested that PMA hydrolase is stored in plasmodia and is released together with the polymer into the culture medium in response to certain environmental conditions.

### 3 Goal of the thesis

Since the discovery of poly(L-malate) in *Physarum polycephalum*, little is known about the genetic regulation of the polymer at the level of its synthesis and degradation. The evidence, that poly(L-malate) is only produced in the plasmodium, suggests a difference in gene expression. The goal of this work was to establish a satisfactory method for the isolation of differentially expressed genes. They should then be characterized and tested by knock-down assays whether they are involved in the biosynthetic pathway of poly(L-malate).

Besides the synthesis, it was of interest to obtain new insight into the degradation of the polymer regulated on the level of polymalatase synthesis. It has been reported that the level of poly(L-malate) in the nuclei was constant and that the excess of the polymer was secreted into the culture medium, where it is degraded by polymalatase. Since the cDNA sequence of the gene encoding for this enzyme has been completely clarified, it is of particular interest to know more about its regulation. Due to the results of western blots with specific antibody against polymalatase, the protein was only found in plasmodia. Thus, it is possible to clarify whether the synthesis of polymalatase is regulated at the transcriptional or translational level.

## II Materials und methods

### 1 Materials

#### 1.1 Apparatus and accessories

apparatus and accessories	manufacturer
accessories for agarose gel	mechanic workshop, University of Regensburg
analytic scales L 610 D	Sartorius
analytic scales LC 2200 P	Sartorius
analytic scales P-1200	Mettler
block heater BT 200	Kleinfeld Labortechnik
blotting apparatus Fastblot	Biometra
centrifuge Superspeed RC2-B	Sorvall
chemoluminescent detection film	Roche
developer OPTIMAX 2010	Protec
fluorescence spectrophotometer F-3000	Hitachi
glass homogenizer	B. Braun
glass potter	B. Braun
incubator with shaker GFL 3033	New Brunswick Science
incubator with shaker GTRO 214	Memmert
incubator with shaker KS 40	Heuser
LightCycler Sytem	Roche
microcentrifuge Biofuge 17 RS	Heraeus Sepatech
microcentrifuge Biofuge 17 RS	Heraeus Sepatech
micro centrifuge 5414	Eppendorf
microinjection apparatus	Leitz
microwave	Siemens
Millipore filtering system	Millipore
PCR cycler DNA Thermal Cycler	Perkin Elmer Cetus

PCR cycler PTC-100™	MJ Research, Inc
pH-meter 766 Calimatic	Knick
picture documentation system	UVP
MultiDoc-It Digital Imaging System	
power supply 800332	CAMAG
power supply Power Pack P25	Biometra
PVDF Immobilon™-P membrane	Millipore
Quarz precision cell (10 mm, 160 µl)	Hellma
shaker G-33-B	New Brunswick Science
SDS gel electrophoresis apparatus	Biometra
SDS gel accessories	Amersham
Speed-Vac RC 10.10	Jouan
Thermomixer	Eppendorf
UV spectrophotometer	Pharmacia Biotech
vortex REAX 200	Heidolph

---

**Table 2** Apparatus and accessories

---

## 1.2 Consumable goods

consumable goods	manufacturer
autoclave tape	A. Hartenstein
autoclavable bags	A. Hartenstein
cryo tubes	A. Hartenstein
cryo box	A. Hartenstein
filter (0,2 µM)	A. Hartenstein
gloves	Kimberly-Clark
microcentrifuge tubes	Eppendorf, Biozym
plastic centrifuge tubes	A. Hartenstein
Pasteur Pipette	BRAND
PCR tubes	Biozym
petri dishes (8.5 and 13.5 cm)	Sarstedt

pH indicator paper	Merck
pasteur pipette	BRAND
pipette tips	Sarstedt; Biozym
plastic cuvettes	Sarstedt
precision pipettes	Gilson

---

**Table 3** Consumable goods

---

## 1.3 Chemicals

---

chemicals	manufacturer
<hr/>	
acetic acid	Roth
agarose NEE0	Roth
ammonium persulfate	Merck
Ampicillin	Roth
Bacto™ agar	Difco
Bacto™ soytone	Difco
Bacto™ tryptone	Difco
Bacto™ yeast extract	Difco
Bromphenol blue	Serve
BSA	Roche
biotin	Merck
citric acid monohydrate	Merck
CTAB	Merck
D(+) glucose monohydrate	Merck
DEPC	Sigma
dextran	Sigma
DTT	Biomol
EDTA	Merck
EGTA	Merck
ethanol	Merck

ethidiumbromid	Merck
FeSO <sub>4</sub> x7H <sub>2</sub> O	Merck
formaldehyd	Sigma
glacial acetic acid	Merck
glycerol	J.T. Baker
glycine	Merck
H <sub>3</sub> PO <sub>4</sub>	Merck
H <sub>2</sub> SO <sub>4</sub>	Merck
HCl	Roth
hemin chloride	Fluka
HEPES	Biomol
IPTG	BioVectra TM
Isopropanol	Merck
K <sub>2</sub> HPO <sub>4</sub>	Merck
KCL	Merck
KH <sub>2</sub> PO <sub>4</sub>	Merck
L-malate, mono sodium salt	Roche
methanol	Merck
β-mercaptoethanol	Merck
MgSO <sub>4</sub> x7H <sub>2</sub> O	Merck
MnSO <sub>4</sub> x7H <sub>2</sub> O	Merck
MOPS	BioMol
Na <sub>2</sub> CO <sub>3</sub>	Merck
NaH <sub>2</sub> PO <sub>4</sub> x2H <sub>2</sub> O	Merck
Na <sub>2</sub> HPO <sub>4</sub> x7H <sub>2</sub> O	Merck
NaCl	Merck
NaS <sub>2</sub> O <sub>5</sub>	Merck
N,N'-dimethyl formamide	Merck
NOWA A	MoBiTec
NOWA B	MoBiTec
Penicillin G	ICN
Ponceau S	Serva
Serva blue	Serva
SDS	Serva

sodium acetate	Merck
streptomycin	Roth
trichloroacetic acid	Merck
thiamine-HCl	Merck
Tris base	Serva
Triton x-100	Sigma
Tween 20	Sigma
X-Gal	Sigma
ZnSO <sub>4</sub> ·7H <sub>2</sub> O	Merck

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**Table 4** Chemicals

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## 1.4 Enzymes, antibodies and vectors

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nucleotides, enzymes, antibodies and vectors	manufacturer/source
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nucleotides:

ATP	MBI Fermentas
CTP	MBI Fermentas
GTP	MBI Fermentas
CTP	MBI Fermentas
dCTP	MBI Fermentas
<sup>33</sup> P-CTP	Amersham
dNTP mix	MBI Fermentas

Dynabeads<sup>®</sup> Oligo(dT)<sub>25</sub>

Dynal

enzymes:

DNase I	Qiagen, MBI Fermentas
inorganic yeast pyrophosphatase	Sigma
Revert Aid H-Minus M-MuLV reverse transcriptase	MBI Fermentas
Ribonuclease H	MBI Fermentas



RNase inhibitor	MBI Fermentas
T4 DNA ligase	Promega
T7 polymerase	MBI Fermentas
<i>Taq</i> polymerase	MBI Fermentas
terminal deoxynucleotidyl transferase	Amersham
restriction enzymes:	
BstU I	NEB
KpnI	NEB
Nco I	NEB
Not I	MBI Fermentas
Spe I	NEB
antibodies:	
BD Living Colors™ A.v. peptide Antibody	BD Biosciences
Anti-rabbit IgG, peroxidase conjugated (secondary antibody)	Pierce
vectors:	
pGEM® T-vector	Promega
pJH40-YFP	University of Freiburg
pJH40-ASEYFP	University of Freiburg

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**Table 5** Nucleotides, enzymes, antibodies and vectors

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## 1.5 Kits

kits	manufacturer
Advantage™ 2 PCR Enzyme System	BD Bioscience
Nucleospin® Plasmid Kit	Machery-Nagel
QIAGEN Plasmid Maxi Kit	Qiagen
QIAquick® Gel Extraction Kit	Qiagen
QIAquick® PCR Purification Kit	Qiagen
Quick Ligation™ Kit	NEB
QuantiTect™ SYBR® Green PCR Master Mix	Qiagen
Rneasy® Mini Kit	Qiagen

**Table 6** Kits

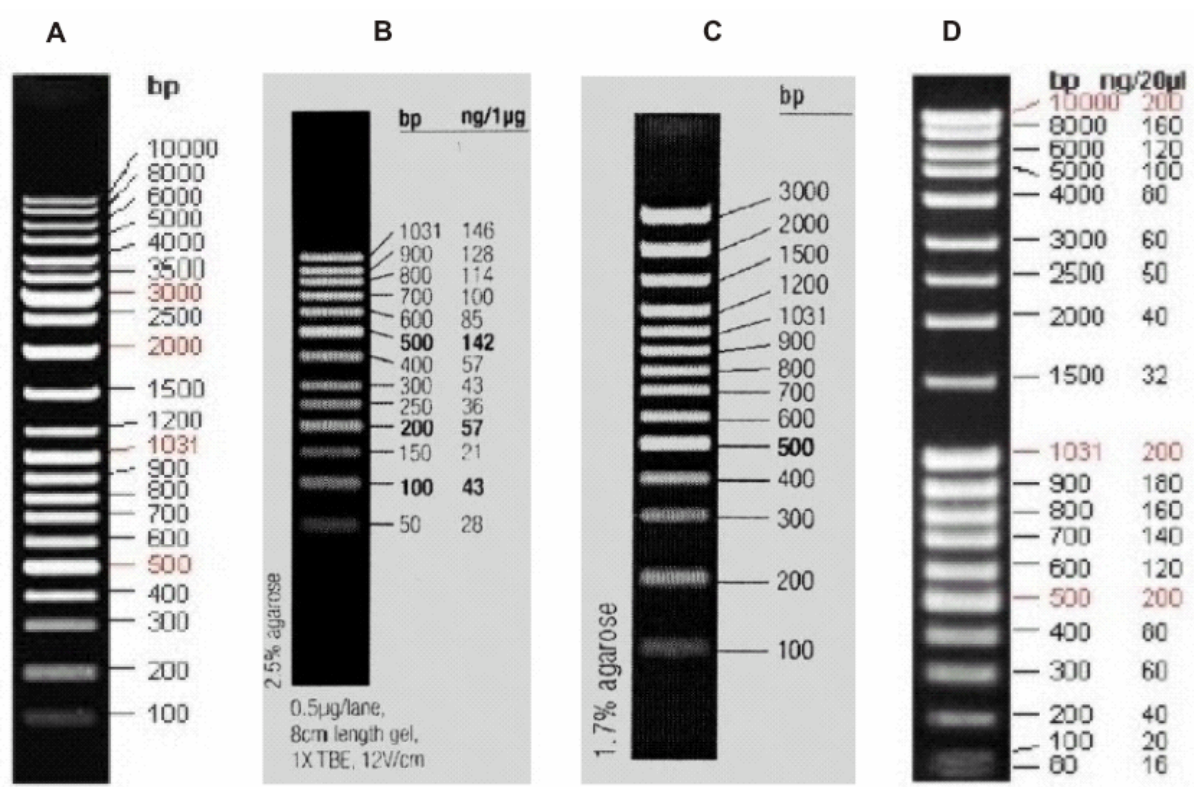
## 1.6 Organisms

organisms	genotyp/marker	source
<i>Escherichia coli</i> DH10B	$\Delta(mrr\text{-}hsd\text{ RMS-}mcrBC)$ <i>mcrA recA1</i>	Bethesda Research Laboratories
<i>Physarum polycephalum</i> : plasmodien: M <sub>3</sub> CVII		ATCC 204388 American Type Culture Collection
amoebae: LU352	matA <sub>2</sub> gadAh npfC5 matB3 fusA1 whiA+ axe	University of Freiburg

**Table 7** Characterization of organisms used

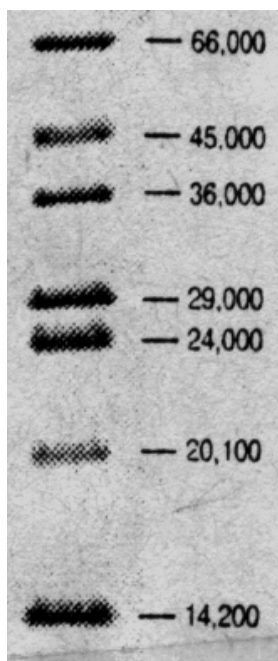
1.7 Standard markers

1.7.1 DNA standard markers



**Figure 4** DNA standard markers. A: GeneRuler™ Ladder Mix. B: GeneRuler™ 50bp. C: GeneRuler™ 100bp. D: MassRuler™

## 1.7.2 Protein standard marker



**Figure 5** Protein standard marker: LWM

## 1.9 Solutions and media for cell culture

### 1.9.1 Solutions and media for plasmodia cultures

#### hemin solution:

50 mg	hemin
5 ml	5 M NaOH
ddH <sub>2</sub> O	to 100 ml

#### MMZ solution:

30 g	MgSO <sub>4</sub> *7H <sub>2</sub> O
4.2 g	MnCl <sub>2</sub> *4H <sub>2</sub> O
1.7 g	ZnSO <sub>4</sub> *7H <sub>2</sub> O
ddH <sub>2</sub> O	to 500 ml

#### N+C medium:

10 g	bacto-tryptone
1.5 g	yeast extract
11 g	D-glucose
3.54 g	citric acid monohydrate
84 mg	FeSO <sub>4</sub> *7H <sub>2</sub> O
2 g	KH <sub>2</sub> PO <sub>4</sub>
0.6 g	CaCl <sub>2</sub> *2H <sub>2</sub> O
10 ml	MMZ solution
ddH <sub>2</sub> O	to 1000 ml

pH was adjusted to 4.6 and 1 ml per 100 ml hemin solution was added before use

N+C agar plates:

4 g agar was added to 100 ml ddH<sub>2</sub>O, and autoclaved, 100 ml sterilized N+C medium and 1 ml sterilized hemin solution were added. The mixture was dispensed into plates.

saltmedium:

3.5 g	citric acid monohydrate
0.085 g	FeSO <sub>4</sub> *7H <sub>2</sub> O
0.6 g	MgSO <sub>4</sub> *7H <sub>2</sub> O
1 g	CaCl <sub>2</sub> *2H <sub>2</sub> O
0.085 g	MnCl <sub>2</sub> *4H <sub>2</sub> O
2 g	KH <sub>2</sub> PO <sub>4</sub>
0.035 g	ZnSO <sub>4</sub> *7H <sub>2</sub> O
ddH <sub>2</sub> O	to 1000 ml

pH was adjusted to 4.6 with 5 M NaOH

## 1.9.2 Solutions and media for amoebae cultures

hemin solution:

Hemin solution for amoebae contained the same components as described in 2.9.1 except that Millipore-H<sub>2</sub>O was used instead of ddH<sub>2</sub>O.

semi-defined medium (SDM):

10 g	Glucose
10 g	bacto-soytone
3.54 g	citric acid monohydrate
2 g	KH <sub>2</sub> PO <sub>4</sub>
1.026 g	CaCl <sub>2</sub> *2H <sub>2</sub> O
0.6 g	MgSO <sub>4</sub> *7H <sub>2</sub> O
34 mg	ZnSO <sub>4</sub> *7H <sub>2</sub> O
42.4 mg	thiamin-HCL
15.8 mg	biotin
Millipore-H <sub>2</sub> O	to 1000 ml

All of the components, except biotin and thiamin, were mixed together. pH was adjusted to 4.6 with 5 M NaOH and the medium was autoclaved. Biotin and thiamin were filter-sterilized and were added separately, like hemin solution, prior to use

DSDM (diluted SDM) plates:

15 g bacto-agar was autoclave together with 1000 ml Millipore-H<sub>2</sub>O. The mixture was allowed to cool down to about 60°C and 65 ml sterile SDM including hemin was added prior dispensing into plates.

SM (Sheffield medium) plates:

15 g	agar
0.7 g	bacto-trytone
0.2 g	Difco yeast extract
0.6 g	D-glucose
0.8 g	NaH <sub>2</sub> PO <sub>4</sub> *H <sub>2</sub> O
0.7 g	Na <sub>2</sub> HPO <sub>4</sub>
15 g	bacto-agar
Millipore-H <sub>2</sub> O	to 1000ml

Formalin-killed bacteria (FKB):

- 1) A 5 ml overnight culture of *E.coli.* was set up by inoculating a single colony from a plate in LB broth and shaken overnight at 37 °C
- 2) 1 ml of the overnight culture was then inoculate in 1 liter of LB broth in a 2 liter flask and was shaken again overnight at 37 °C.
- 3) The culture was spun down at 4000 rpm and 4 °C for 10 min. The pellet was then resuspended in 50 ml FKB buffer.
- 4) The suspension was transferred into a sterile Schott bottle and 4 ml of concentrated formaldehyd solution was added. The bottle was incubated overnight at 4 °C.
- 5) Killed bacteria was pelleted by centrifugation as above and was resuspended in 50 ml FKB buffer.
- 6) 5 ml of 1 M Glycin was added and the suspension was shaken for 1 h at 37°C
- 7) The suspension was centrifuged as step 3 and the pellet was washed with 50 ml FKB buffer. This step was repeated with further 50 ml FKB buffer and the pellet was resuspended in 20 ml sterile Millipore-H<sub>2</sub>O.
- 8) 1 ml aliquots of the suspension were transferred into sterile polypropylene vials and store at 4 °C.
- 9) To test that no live bacteria was present, FKB suspension was streaked on a LB plate and incubate at 37 °C for two days.

FKB buffer:

Na <sub>2</sub> HPO <sub>4</sub>	7 g
KH <sub>2</sub> HPO <sub>4</sub>	3 g
NaCl	4 g
MgSO <sub>4</sub> x7H <sub>2</sub> O	0.1 g
Millipore-H <sub>2</sub> O	to 1000 ml

The solution was autoclaved and aliquots of 250 ml were set up.



1 M glycine solution:

glycine	7.5 g
Millipore-water	to 100 ml

15% glycerol:

15 g glycerol was made up to 100 ml with Millipore-water and was autoclaved before use.

## 1.10 Solutions for analysis of nucleic acids

### 1.10.1 Solutions for mRNA isolation using Dynabeads<sup>®</sup> oligo(dT)<sub>25</sub>

2x binding buffer:

20 mM	Tris-HCl, pH 7.5
10 M	LiCl
2 mM	EDTA

Washing buffer:

10 mM	Tris-HCl, pH 7.5
0.15 M	LiCl
1 mM	EDTA

elution buffer:

Tris-HCl, pH 7.5	10 mM
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DEPC-water:

DEPC	1 ml
ddH <sub>2</sub> O	1000 ml

The suspension was stirred for 30 min at room temperature and was incubated (without shaking) at 37°C overnight. DEPC-water was autoclaved twice before use.

## 1.10.2 Solutions for agarose gel electrophoresis

1% agarose gel:

agarose	1 g
1x TAE	100 ml

The agarose was melted in a microwave and was allowed to cool down to about 60°C before 5 µl of ethidiumbromid stock solution was added.

ethidiumbromid stock solution:

Ethidiumbromid	1 g
ddH <sub>2</sub> O	100 ml

50x TAE buffer:

Tris-base	242 g
glacial acetic acid	57.1 ml
EDTA (0.5 M)	100 ml
ddH <sub>2</sub> O	to 1000 ml

### 1.10.3 Solutions for suppression subtracted hybridization and knock-down assays

#### 4x Hybridization buffer:

NaCl	4 M
HEPES pH 8.3	200 mM
CTAB	4 mM

#### Microinjection buffer:

HEPES	5 mM
EGTA	5 mM
KCl	30 mM
MgCl <sub>2</sub>	1 mM
ATP	1 mM
DTT	1 mM

#### TE-buffer:

Tris-HCl (pH 8.0)	10 mM
EDTA	1 mM

### 1.10.4 Solutions and media for transformation of DNA

#### Ampicillin:

50 mg ampicillin was dissolved in 10 ml ddH<sub>2</sub>O, giving the final concentration of 50 mg/ml. The solution was filter-sterilized and aliquots were stored at -20°C.

IPTG stock solution:

IPTG	1.2 g
ddH <sub>2</sub> O	to 50 ml

The solution was filter-sterilized and stored at -20°C

X-Gal stock solution:

X-Gal	100 mg
N,N'-dimethyl-formamide	2 ml

The solution was covered with aluminium foil and stored at -20°C

LB medium:

Bacto <sup>®</sup> -tryptone	10 g
Bacto <sup>®</sup> -yeast extract	5 g
NaCl	5 g

LB plates with ampicillin:

15 g agar was added to 1 liter of LB medium and was autoclaved. The medium was allowed to cool down to 50°C before ampicillin was added to a final concentration of 100 µg/ml. The mixture was immediately poured into petri dishes. The plates were stored at 4°C for up to 2 months.

LB plates with ampicillin /IPTG/X-Gal:

The LB plates with ampicillin were made as above. 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal were spread over the plates. The plates were allowed to absorb for 30 min at 37°C before use.

SOC medium:

Bacto <sup>®</sup> -tryptone	2 g
acto <sup>®</sup> -yeast extract	0.5 g
1 M NaCl	1 ml
1M KCl	0.25 ml
2M Mg <sup>2+</sup> stock	1 ml
2 M glucose	1 ml

First, tryptone, yeast, NaCl and KCl were dissolved in 97 ml ddH<sub>2</sub>O. The mixture was autoclaved and allowed to cool down before sterile Mg<sup>2+</sup> and glucose were added. The medium was made up to 100 ml with sterile, distilled water and was then filter-sterilized.

2M Mg<sup>2+</sup> stock:

MgCl <sub>2</sub> ·6H <sub>2</sub> O	20.33g
MgSO <sub>4</sub> ·7H <sub>2</sub> O	24.65g

The stock solution was made up to 100 ml with ddH<sub>2</sub>O and filter-sterilized.

## 1.11 Solutions for SDS-PAGE and Western Blotting

### 1.11.1 Solutions for cell lysis and SDS electrophoresis

buffer for total cell lysis:

Tris-HCl (pH 7.5)	50mM
Na <sub>2</sub> S <sub>2</sub> O <sub>5</sub>	5 mM
EGTA	50 mM
MgCl <sub>2</sub>	10 mM

NaCl	300 mM
riton X-100	0.5%

1/25 volume of protease inhibitor cocktail and 1/1000 volume mercaptoethanol were added before use.

Bradford reagent stock solution:

Serva Blue G	100 mg
95% ethanol	50 ml
H <sub>3</sub> PO <sub>4</sub>	100 ml
ddH <sub>2</sub> O	850 ml

12 % separation gel:

40% acrylamide mix	1800 µl
1,5 M Tris HCL (pH 8,8)	1,5 ml
ddH <sub>2</sub> O	2,6 ml
10 % SDS solution	60 µl
10 % APS solution	30 µl
TEMED	5 µl

5 % stacking gel:

40 % acrylamide mix	310 µl
0,5 M Tris HCl (pH 8.8)	500 µl
ddH <sub>2</sub> O	1645 µl
10 % SDS	25 µl
10 % APS	15 µl
TEMED	2,5 µl

## 1.11.2 Solutions for Western Blotting:

Western Blotting buffer:

methanol	20%(v/v)
Tris-base	50 mM
glycine	40 mM

2x SDS gel loading buffer:

Tris-HCl pH 6.8	90 mM
glycerol	19% (w/v)
SDS	2% (w/v)
$\beta$ -mercaptoethanol	0.7 mM
bromphenol blue	0.015 mM

SDS gel running buffer:

Tris-base	25 mM
glycine	192 mM
SDS	0.1% (w/v)

pH was adjusted to 8.8 with concentrated HCl.

Ponceau staining solution:

Ponceau S	2%(w/v)
TCA	30%(v/v)

1x PBS:

KH <sub>2</sub> PO <sub>4</sub>	2 mM
Na <sub>2</sub> HPO <sub>4</sub>	10 mM
NaCl	137 mM
KCl	2.7 mM

pH was adjusted to 7.4 with concentrated HCl.

PBS-Tween:

1xPBS	1000 ml
Tween-20	2 ml

## 1.12 Solutions for quantitative analysis of PMLA

glycine-hydrazine buffer:

glycine	0.76 M
hydrazine	0.5 M

pH was adjusted to 9 with 5 M NaOH and was stored at 4°C.

10x L-malate stock solution:

L-malate mono sodium salt	100 mg
ddH <sub>2</sub> O	10 ml

Aliquots were set up and stored at –20°C



malate dehydrogenase stock solution:

The stock solution was diluted 10-fold with 50 mM Tris-HCL buffer (pH7.5) giving an activity of 0.6 units/ml

NAD<sup>+</sup> solution (40 mM):

NAD <sup>+</sup>	133 mg
ddH <sub>2</sub> O	5 ml

The solution was stored at 4°C for up to 2 weeks.

## 2 Methods

### 2.1 Cell culture

#### 2.1.1 Cultivation of plasmodia

##### 2.1.1.1 Cultivation of microplasmodia

Microplasmodia of the strain M<sub>3</sub>CVII were grown in the dark at 24 °C with constant stirring in 100ml N+C medium, supplemented with 1 ml sterile hemin solution. After 1-2 days the microplasmodia were harvested. As plasmodia develop to spherules in starvation, culturing of microplasmodia in the same medium for longer than 3 days should be avoided. Usually 2 ml of microplasmodia was inoculated in fresh medium every two days. For long period, it is recommended to maintain microplasmodia as spherules.

### 2.1.1.2 Induction of Spherules

To obtain spherules, 2 days old microplasmodia were transferred to a non-nutrient saltmedium and were shaken in the dark at 24 °C for 2 days. After replacing with a fresh saltmedium the cells were continuously incubated at 24 °C with stirring. After further 3 days spherules were visible. Spherules in salt medium, stored at 4 °C will remain viable for several months. For a longer storage, it is recommended to drop spherules on sterile Whatmann filters and store them at 4 °C. Plasmodia were induced by putting the spherules into a fresh liquid medium or on a agar plate. From the agar plate a small piece were cutted and inoculated into a liquid culture medium.

### 2.1.1.3 Cultivation of macroplasmodia

Macroplasmodia were obtained by placing 300 µl of concentrated microplasmodial suspension on a 9 cm Petri dish or 400 µl of microplasmodia on a 13.5 cm petri dish filled with N+C medium containing 2% agar. Macroplasmodia were growth in the dark at 24 °C.

## 2.1.2 Cultivation of amoebae

### 2.1.2.1 Growth of amoebae on DSDM agar plates

DSDM plates were inoculated with  $2-3 \times 10^5$  amoebal cysts in glycerol and 200 µl diluted formalin-killed bacteria (diluted with millipore water 1:1). The plates were then incubated at 24°C for 48 h and transferred at 30 °C. After 4 days at 30 °C the plates became confluent and were harvested. It is recommended to maintain amoebal strains as glycerol stocks at -80°C .

#### 2.1.2.2 Preparation of amoebal stock culture

Amoebae were inoculated from a glycerol stock culture on DSDM agar plates as above (2.1.2.1). The plates were incubated at 24 °C for 48 h to facilitate hatching of amoebae from cysts. After 48 h the plates were transferred to 30 °C and incubated for further 8 days. After then, most of the amoebae were transformed to cysts. These plates can now be stored at 4 °C for 3 months and used as stock cultures. Alternatively they can be used to prepare glycerol stocks. For this purpose the 10 days old plates were flooded with 10 ml of 15 % (w/v) glycerol and scraped with a glass pipet. 1 ml aliquots were then freeze-dried at – 80 °C. Cysts stored at that temperature remain viable for many years and can be thawed repeatedly.

#### 2.1.2.3 Growth of amoebae in axenic liquid medium

Amoebae were inoculated from a glycerol stock culture on DSDM agar plates as above (2.1.2.1). The plates were incubated at 24 °C for 48 h and transferred to 30 °C for further 8 days. Amoebae were then inoculated by toothpick onto SM-plates, containing 250 µg/l streptomycin, 250 µg/l penicillin and 200µl diluted formalin-killed bacteria. The plates were then incubated at 24 °C for 48 h and 30 °C for 4 days. After then, the plates were flooded with 10 ml of SDM including 1 % hemine and incubated for 1 h, no scraping. Then suspension is sucked off with a 10 ml pipet and made up to 50 ml with SDM containing hemine and 250 µg/l each streptomycin/penicillin. The suspension were transferred to a 500 ml flask and incubated on a shaker (ca. 150 rpm) at 30 °C. Growth rates were initially slow. When the growth rate increased to a doubling time of around 18-24 h, which usually occurs 1-2 weeks after the first inoculation, 5 ml aliquots are inoculated into a fresh 45 ml of SDM including hemine. Antibiotics were omitted after the initial inoculation. To prevent the commitment of amoebae to plasmodia, amoebae were subcultured before the cell density exceeded  $10^7$ /ml. However, more than 30 subcultures should be avoided.

## 2.2 Isolation of nucleic acids

### 2.2.1 Isolation of total RNA

- 1) Cell lysis: 450  $\mu$ l of Buffer RLT was added to a maximum of 100 mg of frozen cells and was vortexed vigorously.
- 2) Homogenization: The lysate was applied to the QIAshredder spin column and was centrifuged at 14000 rpm for 2 min.
- 3) Ethanol precipitation: The flow-through was carefully transferred to a new tube and was mixed well with 0.5 volumes of 100% ethanol. The sample was then applied to a RNeasy column and centrifuged at 10,000 rpm for 15 sec.
- 4) Washing: To wash the membran, 350  $\mu$ l of Buffer RW1 was added and the column was centrifuged for 15 sec at 10,000 rpm.
- 5) On-column DNase digestion: 10  $\mu$ l of DNase I stock solution was added to 70  $\mu$ l Buffer RDD and mixed gently by inverting the tube. The mix was directly pipetted onto the RNeasy silica-gel membrane. The column was then incubated at room temperature for 15 min.
- 6) Washing: The RNeasy column was transferred into a new collection tube and 500  $\mu$ l Buffer RPE was added. The column was centrifuged for 15 s at 10,000 rpm. The washing step was repeated once with further 500  $\mu$ l Buffer RPE. To dry the membrane completely, the column was centrifuged at full speed for 1 min.
- 7) Elution: To elute RNA, the RNeasy column was transferred to a RNase-free 1.5 ml tube and 50  $\mu$ l RNase-free water was added. The column was then centrifuged at 10,000 rpm for 1 min. To obtain a higher total RNA concentration, the elution step was repeated by using the first eluate.

## 2.2.2 Poly A<sup>+</sup> mRNA isolation from total RNA using Dynabeads<sup>®</sup> Oligo(dT)<sub>25</sub>

### 2.2.2.1 Principle

Dynabeads Oligo (dT)<sub>25</sub> are uniform, superparamagnetic (2,8 µm diameter) with 25 nucleotid-long chains of deoxythymidines covalently linked to their surfaces. Dynabeads Oligo (dT)<sub>25</sub> are designed for rapid isolation of poly A<sup>+</sup> RNA either from total RNA or directly from crude extracts. The use of Dynabeads Oligo (dT)<sub>25</sub> relies on base pairing between the poly A tail of most messenger RNA and the oligo dT sequences. The binding capacity of the Dynabeads Oligo (dT)<sub>25</sub> is 2 µg polyadenylated mRNA per mg Dynabeads.

### 2.2.2.2 Procedures

- 1) The Dynabeads Oligo (dT)<sub>25</sub> was resuspended by gently flicking the tube and 85 µl of Dynabeads Oligo (dT)<sub>25</sub> from the stock suspension was transferred to an RNase-free 1.5 ml microcentrifuge tube.
- 2) The tube was then placed in magnet stand (DynaL MPC-E-1) for 30 sec. After the supernatant discarded, the tube was removed from the magnet stand and the Dynabeads was resuspended in 100 µl binding buffer.
- 3) The tube was placed in magnet stand again to remove the binding buffer.
- 4) The Dynabeads was resuspended again in 100 µl binding buffer. 25 µg Total RNA was adjusted to 100 µl with DEPC-treated water and was heat at 65°C for 2 min.
- 5) The total RNA was then mixed to the Dynabeads and the suspension was incubated on a rotating mixer for 5 min to anneal RNA.
- 6) The tube was placed in the magnet stand for 30 sec. The supernatant was removed and the Dynabeads was washed twice with 200 µl washing buffer.
- 7) 15 µl of elution buffer was added and the tube was incubate at 65 °C for 2 min. The tube was placed in the magnet stand again and the supernatant containing mRNA was transferred to a new RNase-free tube.

- 8) To eliminate any ribosomal RNA contamination, the eluted mRNA was reextracted. First, the Dynabeads was washed twice with 200 µl washing Buffer. The beads were then resuspended in 60 µl binding buffer. After incubation on a roller for 5 min at room temperature, the Dynabeads Oligo (dT)<sub>25</sub>/mRNA was washed twice with 200 µl washing buffer and the mRNA was eluted as above.

### 2.2.3 Isolation of DNA using QIAquick PCR Purification Kit

- 1) DNA binding: 5 volumes of Buffer PB was added to 1 volume of the PCR sample and mixed. The QIAquick spin column was loaded with the sample and was centrifuged for 1 min.
- 2) Washing: 0.75 ml of Buffer PE was added and the column was centrifuged for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min.
- 3) To elute DNA, 50 µl of Buffer EB was added and the column was centrifuged for 1 min. If higher DNA was desired, 30 µl Buffer EB was added and the column was incubated at room temperature for 1 min. The column was then centrifuged as above.

### 2.2.3 Isolation of DNA from agarose gel using QIAquick

#### Gel Extraction Kit

All centrifuge steps were at 13,000 rpm and at room temperature.

- 1) The DNA fragment was excised from the agarose gel and weighed. 3 volumes of Buffer QG were added to 1 volume of gel. The gel slice was incubated at 50 °C for 10 min. To support dissolving, the tube was mixed by vortexing every 2-3 min.

- 2) To increase the yield of DNA fragments <500 bp, 1 gel volume of isopropanol was added to the sample and mixed. If the fragment was >500 bp this step was skipped as addition of isopropanol has no effect on yield.
- 3) The sample was loaded to a QIAquick spin column and the column was centrifuged for 1 min.
- 4) 0.75 ml of Buffer PE was added and the column was centrifuged for 1 min. The flow-through was discarded and the column was centrifuged for an additional 1 min.
- 5) 50 µl of Buffer EB was added and the column was centrifuged for 1 min to elute DNA. Alternatively, to increase DNA concentration, 30 µl Buffer EB was added and the column was incubated at room temperature for 1 min. The column was then centrifuged as above.

### 2.2.5 Isolation of Plasmid DNA using Nucleospin<sup>®</sup> Plasmid Kit

All centrifugation steps were at 13,000 rpm and at room temperature.

- 1) Cell lysis: 4 ml of a saturated *E. coli* LB culture was centrifuged for 30s and the pellet was mixed with 250 µl buffer A2 by vigorous vortexing. 250 µl buffer A2 was added and gently mixed by inverting the tube 6-8 times. The tube was then incubated at room temperature for 5 min. 300 µl buffer A3 was added and gently mixed as above.
- 2) Clarification: The lysate was centrifuged for 10 min and the supernatant was loaded onto the column. The column was then centrifuged for 1 min.
- 3) Washing: 500 µl prewarmed (at 50 °C) buffer AW was added and the column was centrifuged again for 1 min. 600 µl buffer A4 was added and the column was centrifuged for 1 min. To dry the silica membrane completely, the column was centrifuged again for 2 min.
- 7) Elution of DNA: 25 µl of prewarmed (at 70°C) buffer AE was added. The column was incubated for 3 min and was then centrifuged for 1 min. The elution step was repeated with further 25 µl prewarmed buffer AE, to obtain higher yield of DNA.

### 2.2.6 Isolation of plasmid DNA using QIAGEN Plasmid Maxi Kit

- 1) Cultivation of bacteria cells: A single colony was inoculated in 5 ml LB medium containing ampicillin and was incubated for 8h at 37°C with vigorous shaking (~250 rpm). 1 ml of this starter culture was inoculated in 500 ml LB medium with ampicillin and was shaken as above for 16 h.
- 2) Cell lysis: The bacterial cells were harvested by centrifugation at 6000 rpm in a Sorvall GSA rotor at 4°C for 15 min. After the pellet was resuspended in 10 ml Buffer P1, 10 ml of Buffer P2 was added. The mixture was then gently mixed by inverting 4-6 times and was incubated at room temperature for 5 min. 10 ml of ice chilled Buffer P3 was added. The suspension was immediately mixed by inverting 4-6 times and was incubated on ice for 20 min.
- 3) Clarification of the lysate: The lysate was centrifuged at 13,000 rpm at 4 °C for 30 min. The supernatant containing plasmid DNA was promptly removed and centrifuged again for 15 min.
- 4) Column equilibration: A QIAGEN-tip 500 was equilibrated by applying 10 ml Buffer QBT. The column was then allowed to empty by gravity flow
- 5) DNA binding: The supernatant from step 3 was then loaded to the column and was allowed to flow through. The QIAGEN-tip 500 was washed twice with 30 ml Buffer QC.
- 6) Elution and DNA precipitation: DNA was then eluted with 15 ml Buffer QF and was precipitated by adding 10.5 ml of room-temperature isopropanol. The sample was then mixed and centrifuged immediately at 11,000 rpm in a Sorval SS-34 rotor at 4°C for 30 min. The pellet was washed with 5 ml of room-temperature 70% ethanol and centrifuged as above for 10 min. The pellet was air-dried for 5-10 min and was redissolved in 200 µl of 10 mM Tris-HCl (pH 8,5).



## 2.3 Analysis and amplification of nucleic acids

### 2.3.1 Quantification of nucleic acids

The concentration and purity of nucleic acids can be determined by measuring the absorbance at 260 nm and 280 nm in a UV spectrophotometer. Absorbance readings at 260 nm measure concentration of the nucleic acids and should be between 0.1 and 1 to ensure reliable quantification. Spectrophotometric conversion values of nucleic acids are listed in Table 9. An absorbance of 1 unit at 260 nm, e.g., when measuring RNA samples, corresponds to 40 µg RNA per ml. The ratio of the readings at 260 nm and 280 nm ( $A_{260}/A_{280}$ ) provides an estimate of the purity of nucleic acids with respect to contaminants that absorb in the UV, such as protein. Pure RNA, e.g., has an  $A_{260}/A_{280}$  ratio of 1.8-2.1.

1 $A_{260}$ unit	concentration (µg/ml)
double-stranded DNA	50
single-stranded DNA	33
RNA	40

**Table 8:** Spectrophotometric conversion for nucleic acids

Concentration of nucleic acids can be calculated as following:

$$\text{concentration of DNA, RNA} = 1 A_{260} \text{ unit} \times A_{260} \times \text{dilution factor}$$

However,  $A_{260}$  readings <0.1 lead to considerably lower reproducibility. Thus, when working with small amounts of DNA, quantification by agarose gel, using a standard marker, may be more reliable.

### 2.3.2 Polymerase chain reaction (PCR)

- 1) Thermal cycler was equilibrated to 94°C. This manual “hot start” was performed to support the specificity of the amplification reaction. For each PCR sample the following reagents were combined into a PCR tube chilling on ice:

components	volumes( $\mu$ l)
sterile Millipore-water	to 50
10 PCR buffer with $(\text{NH}_4)_2\text{SO}_4$	5
25 mM $\text{MgCl}_2$	4
10 mM dNTP mix	1
10 $\mu$ M forward primer	1
10 $\mu$ M reverse primer	1
DNA	variable
<i>Taq</i> polymerase	0.5
final volume	50

- 3) The tubes were transferred into the preheated thermal cycler and the amplifications reactions were performed using the following cycling parameter:

initial denaturation: 94°C for 2 min

cycle 35x:

denaturation: 94°C for 30 s

annealing of primers: 54°C for 30 s

primer extension: 72°C for 2 min

final extension: 72°C for 7 min

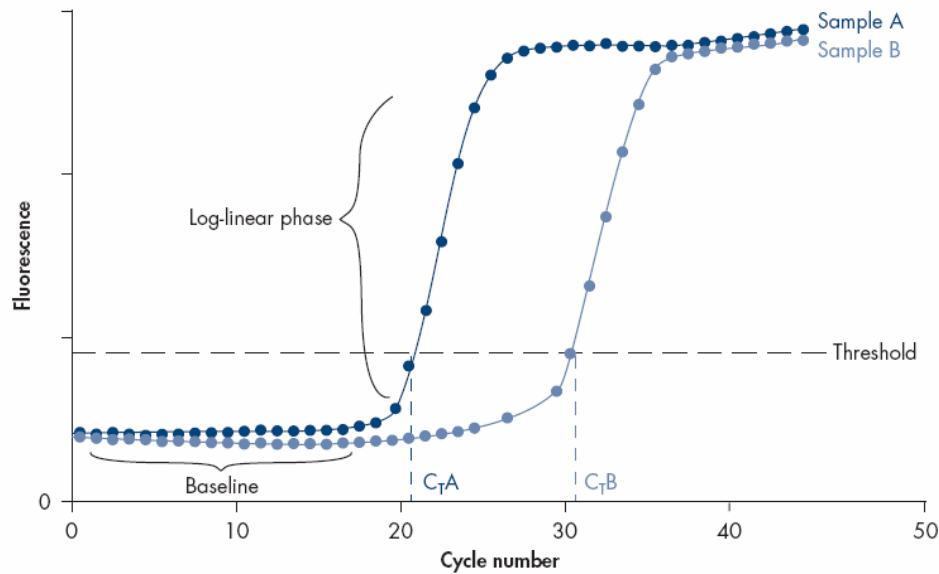
indefinite hold: 0°C, until samples were removed

Most of amplification reactions in this work, except noted, were performed as described here. The amount of the starting material and the primers used varied and were given in details at each experiment.

### 2.3.3 Real time PCR

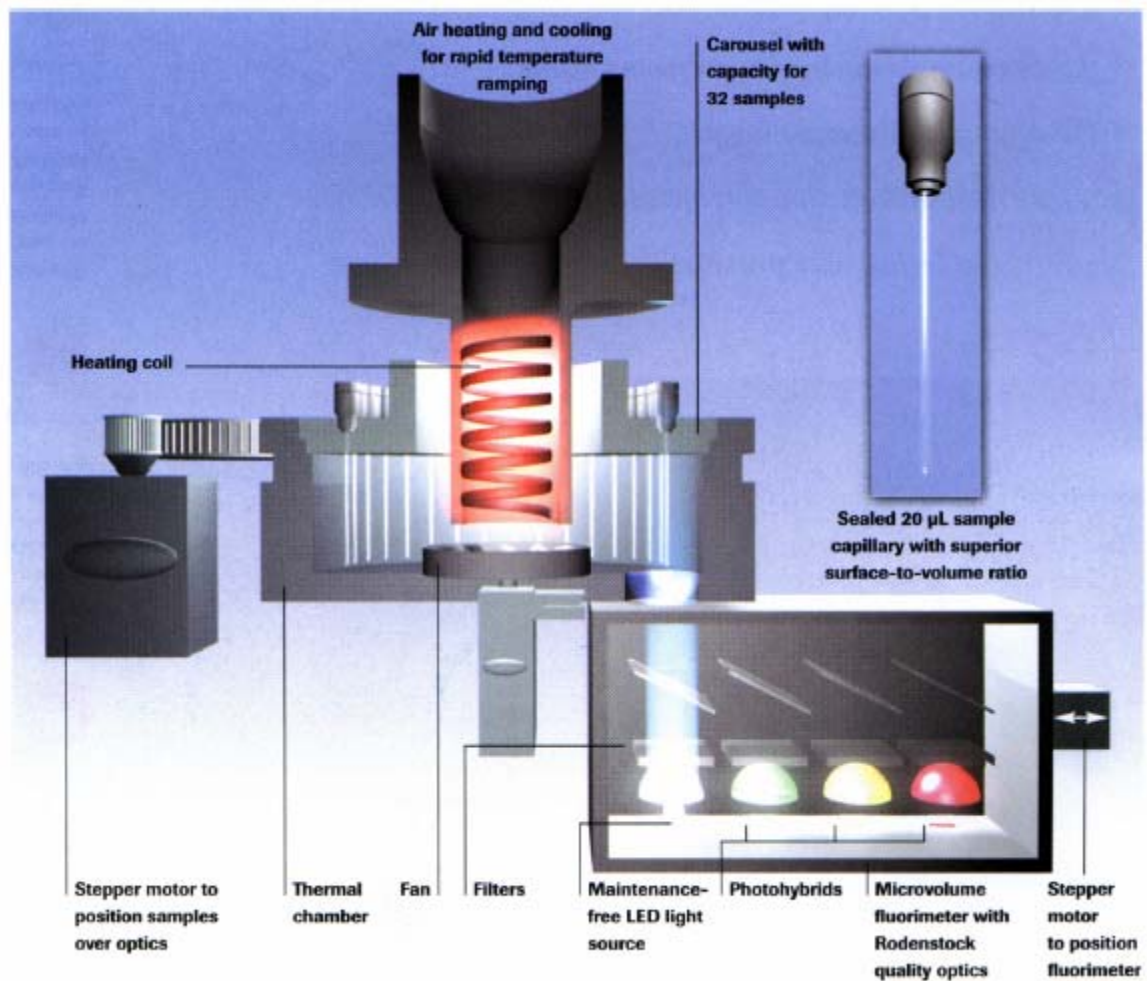
#### 2.3.3.1 Principle

The concentration of a target DNA relative to a standard can be estimated by using a polymerase chain reaction. However the nature of PCR itself may cause some difficulties. As PCR is an exponentially process, small differences in efficiency at each cycle can lead to large differences in the yield of the amplified product. The amount of PCR product increases logarithmically in the first PCR cycles before the plateau is reached. For this reason, methods that quantify the amount of amplified DNA after these first cycles are regarded as unreliable. In recent years, more sophisticated methods have been developed in which amplified DNA is quantified during the exponential phase of the PCR. One of these methods is the so-called Real time PCR. In a real time PCR a fluorescence-detecting thermocycler is used to amplify nucleic acid sequences and measure their concentration simultaneously. The instrument plots the rate of increasing fluorescence against the number of cycles. The greater the initial concentration of target sequences, the fewer the number of cycles required to achieve the threshold of amplification. The initial concentration of target sequences can therefore be expressed as the cycle number required to observed the first detectable increase in fluorensence (the so-called threshold cycle,  $C_T$ , see Figure 6). A plot of  $C_T$  against the  $\log_{10}$  of the amount of standard samples results in a straight line. The initial amount of the target is calculated by interpolation into this standard curve.



**Figure 6** Amplification plots from 2 samples: As the  $C_T$  of sample A is lower than that of sample B, sample A contains higher amount of starting material [QIAGEN, Quantitect™ SYBR® Green PCR Handbook]

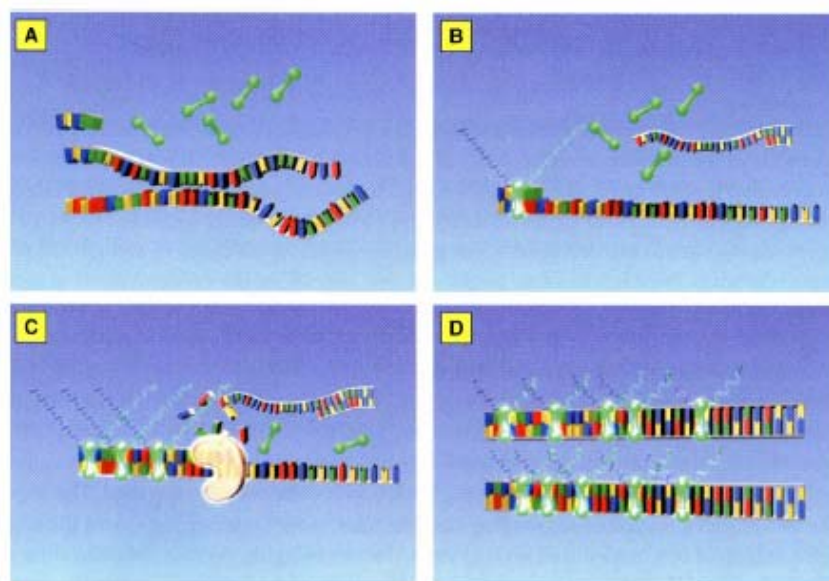
One of the real time PCR instruments is the LightCycler System (Figure 7). In the LightCycler, the amplification reactions are set up in borosilicate glass capillaries that are placed in a carousel. The optical properties of borosilicate glass enable the capillaries to be used as cuvettes for fluorescence measurements. The optical unit of the LightCycler has three detection channels which measure emitted light at three different wavelengths and a light-emitting diode (LED) as the light source. Blue light from the LED is focused on the capillary tip and excites the fluorophore. The emitted fluorescent light is conducted back to the optical unit. A set of filters and mirrors separates the emitted light into different wavelengths that can be detected in one of the three channels. The fluorescence data stream is converted into an amplification plot, giving cycle-by-cycle monitoring.



**Figure 7** Schematic of the LightCycler System [Roche Applied Science]

Cycle-by-cycle monitoring precisely identifies the cycles in which the PCR is in the log-linear phase, with the PCR product doubling with each cycle. In this phase, the signal is easily distinguished from the background signal, providing accurate information about the starting concentration of the target sequence. Several fluorescence formats are available for correlating the amount of PCR product. Straining with ethidium bromid is the most widespread method. However, due to its low sensitivity and specifity, ethidium bromid is not used in the LightCycler. Using double-stranded DNA binding dye, SYBR Green I, provides more specificity since it only fluorescences when bound to dsDNA. During the various stage of PCR, different intensities of fluorescence signals are detected, depending on the amount of dsDNA that is present. After denaturation all DNA becomes single-stranded (Figure 8 A). At this stage, SYBR Green I will not bind to DNA, thus the intensity of fluorescence

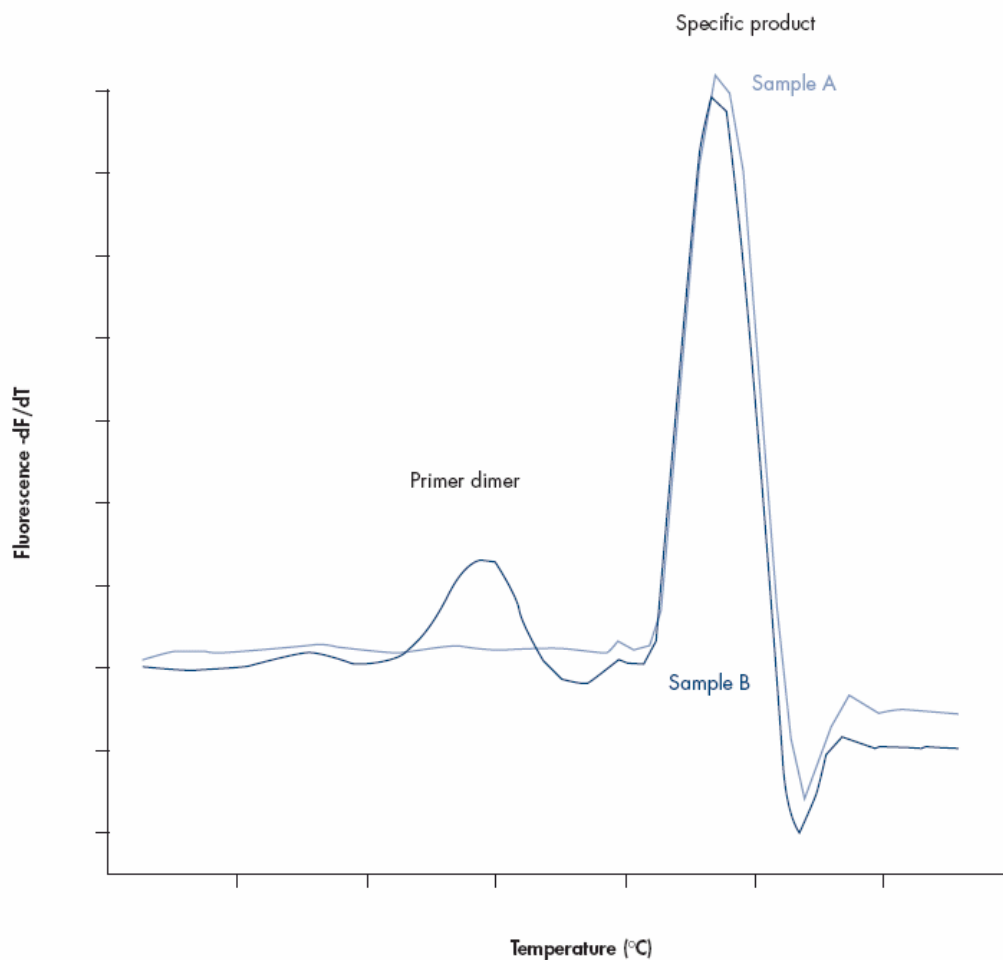
signals is low. During annealing, the primer hybridizes to the target sequence, resulting in dsDNA, to which SYBR Green I can bind. Thus, the intensity of fluorescence is increased during this stage (Figure 8 B). In the elongation phase, the PCR primers are extended and more SYBR Green I dye can bind (Figure 8 C). At the end of the elongation phase, all of the DNA becomes double-stranded, and a maximum amount of dye is bound (Figure 8 D). The fluorescence is recorded at the end of the elongation phase at 530nm and increasing amount of PCR product can be monitored.



**Figure 8** Monitoring of PCR with the SYBR Green I dye [Roche Application Manual, [www.roche-applied-science.com](http://www.roche-applied-science.com)]

Furthermore, the Light cycler can also provide sequence confirmation of the amplified product, through a function called melting curve analysis, performed after PCR. Each dsDNA product has its own specific melting temperature, which is defined as the temperature at which 50% of the DNA becomes single stranded, and 50% remains double stranded. The most important factors that determine the  $T_m$  are the length and the GC content of the fragment. At the end of the PCR run, the temperature in the thermal chamber is slowly raised. At low temperatures, all PCR products are double-stranded, allowing the SYBR Green dye to bind to them. At high temperature, the fluorescence of the SYBR Green I bound to ds amplicons drops sharply as PCR products are denatured. The decrease of fluorescence ( $dF$ ) is plotted

against intervals of increasing temperature ( $dT$ ). The detection systems calculate the first negative derivative ( $-dF/dT$ ) of the curves, resulting in curves with peaks at the respective melting temperatures. Curves with peaks at a  $T_m$  lower than that of the specific product indicate the formation of primer-dimers (Figure 9). Diverse peaks with different  $T_m$ s or plateaus indicate nonspecific products.



**Figure 9** Melting curve analysis of 2 samples: Sample A shows only one peak indicating a specific amplification product, while sample B includes an additional peak at a lower temperature resulting from amplification of primer-dimers. [QIAGEN, Quantitect<sup>TM</sup> SYBR® Green PCR Handbook]

Target nucleic acids can be quantified using either absolute or relative quantification. Absolute quantification determines the absolute amount of the target, expressed as copy number or concentration, by using an external standard. This standard usually contains sequence that is identical or highly similar to the target sequence, whereas the primer binding sites must be the same. These conditions ensure equivalent amplification efficiencies of standard and target molecules, which is essential for this approach. In a relative quantification, the ration between the

amount of target and reference molecules is quantified. The most common application of relative quantification is analysis of gene expression in different samples. However, for reliable results, the expression level of the reference molecule must be constant under different experimental conditions, or in different stage of the same organism. Therefore, a housekeeping gene is usually used as a reference molecule. For both absolute and relative quantification, to generate a standard curve, at least five different concentrations of the standard should be measured, with the amount of target falling within the range tested. Furthermore, for the best efficiency the expecting size of amplified products should be 100-150 bp.

### 2.3.3.2 Experimental procedure

#### 2.3.3.2.1 Absolute quantification

- 1) To analyse the expression level of PMLA hydrolase at the different stages of the life cycle, a PCR fragment containing the target sequence was used as external standard for the absolute quantification. To create the external standard, two samples of PCR were performed in a conventional thermal cycler. The samples contained components shown in 2.3.2.2 including 40 ng of cDNA (derived from the plasmodium), PMA-N1 (forward primer) and L2.2 RV (reverse primer). 35 cycles of PCR (annealing temperature = 55°C) were performed as described in 2.3.2.2 and the amplified products were electrophoresed on a 2% agarose gel.
- 2) DNA fragments were purified together in one spin column, to increase the concentration of the yield.
- 3) Concentration of purified DNA was estimated by UV spectrophotometry and 16 ng DNA was ligated with 50 ng of pGEM T vector
- 4) The plasmid was then transformed into DH10B competent cells (see 2.4) and was isolated using Nucleospin Plasmid Kit.
- 5) Six samples of NcoI/SpeI digestion with 2 µg Plasmid DNA each sample were set up to isolate the insert containing the desired PCR products. The digestions were performed at 37°C for 1.5 h. The samples were then



analysized on a 2% agarose gel. The appropriate fragments were isolated with one spin column and the yield was estimated.

- 6) The purified DNA was diltuted 1:100,000. Five different concentrations from this diluted sample were set up and were applied for the standard curve in the LightCycler System.
- 7) Two samples of master mix were prepared according to Table 9 .

	master mix 1	master mix 2
2x QuantiTect SYBR Green	80 µl	90 µl
PCR Master Mix		
10 µM L2.1 F (forward primer)	8 µl	9 µl
10 µM L2.1 RV (reverse primer)	8 µl	9 µl
RNase free-water	48 µl	
Total volumne	144 µl	108 µl

**Table 9** Preparation of PCR master mix for absolute quantification

- 8) To perform a standard curve, 18 µl of master mix 1 was aliquoted into 6 PCR capillaries. 2 µl of five different dilutions of standard DNA (step7) or 2 µl of RNase-free water (negativ control) was added, giving the final volume of 20 µl each capillary.
- 9) For each target sample (amoebae, plasmodium and spherules), 3 tubes were set up with 12 µl of master mix 2, 40 ng of single-stranded cDNA and the appropriate amount of RNase-free water to give the final volume of 20 µl each tube. The whole content of the samples were dispensed into capillaries.
- 10)The real-time PCR was performed using the following cycler conditions:

15 min 95°C activation of HotStarTaq DNA Polymerase  
cycling: 35x  
15 s 94°C denaturation

20s 58°C      annealing  
20s 72°C      extension

### 2.3.3.2.2 Relative quantification

To confirm that the knock-down experiments were successful, the amount of target sequences after injection was determined using relative quantification.

- 1) For each target sequence a standard curve was performed with cDNA from the sample without injection. For each standard curve, 5 different concentrations of the standard DNA were used for the PCR. To create standard curves, three samples of master mix were prepared according to table 10 .

	master mix 1	master mix 2	master mix 3
2x SYBR Green Master Mix	50 µl	50 µl	50 µl
10 µM pP35 F3	5 µl		
10 µM pP35 R2	5 µl		
10 µM NKA 8 F2		5 µl	
10 µM NKA 8 R2		5 µl	
10 µM NKA 48 F1			5 µl
10 µM NKA 48 R2			5 µl
RNase free-water	30 µl	30 µl	30 µl

**Table 10** Preparation of master mix for relative quantification

- 2) For each standard curve, a negative control containing RNase-free water instead of DNA, was performed. 18 µl of master mix samples was aliquoted into capillaries and 2 µl of standard cDNA or 2 µl of water was added, giving the final volume of 20 µl each capillary.

- 3) 2 µl of cDNA derived from the samples with unknown amount of targets was dispensed into PCR capillaries. 10 µl of SYBR Green PCR Master Mix and 6 µl of RNase-free water were added to each capillaries.
- 4) The real-time PCR was performed under the same conditions as above (2.3.3.2.2 step 10).

## 2.3.4 RT-PCR

### 2.3.4.1 Principle

Reverse transcriptase-PCR is a sensitive and versatile method for amplification cDNA copies of RNA. RT-PCR is used to retrieve the 5' and 3'-ends of mRNAs and to generate cDNA libraries from very small amounts of mRNA. The first step of RT-PCR is the conversion of RNA to a single-stranded cDNA template (so called first-strand cDNA synthesis). The conversion begins with the annealing of oligodeoxynucleotide primer, which is then extended by an RNA-dependent DNA polymerase (reverse transcriptase). The created cDNA copy can then be amplified by a conventional PCR. The first-strand cDNA synthesis can be primed by using either a gene-specific primer, oligo(dT), or random hexanucleotides. It is desirable to generate a first-strand synthesis that is as long as possible and contains a high proportion of specific product. Hence, synthetic gene-specific oligonucleotides are usually the primers of choice. If the sequence of the target RNA is unknown, oligo(dT), which binds all mRNAs containing poly(A)<sup>+</sup> tails, is the next best option. In general, random hexamers, which have no specificity are only used when the other methods fail.

#### 2.3.4.2 First-strand cDNA synthesis using oligo(dT) primer

- 1) The following was added to a nuclease-free thin-walled PCR tube:

components	amount
oligo(dT) primer	10 pmoles
total RNA	0.5-2 $\mu$ g
DEPC-treated water	to 9.5 $\mu$ l
total volume	9.5 $\mu$ l

- 2) To denature RNA, the mixture was incubated for 10 min at 70°C and was then chilled on ice for 1 min. After the contents was collected by brief centrifugation, the following reagents was added in the order given:

Component	Volume ( $\mu$ l)
5x reaction buffer	4
10 mM dNTP mix	2
(0.1 M) DTT	2
(10 mg/ml)BSA	0.4
100 mM) MgCl <sub>2</sub>	0.6
(40 U/ $\mu$ l) RNasin	0.5
total volume	9.5

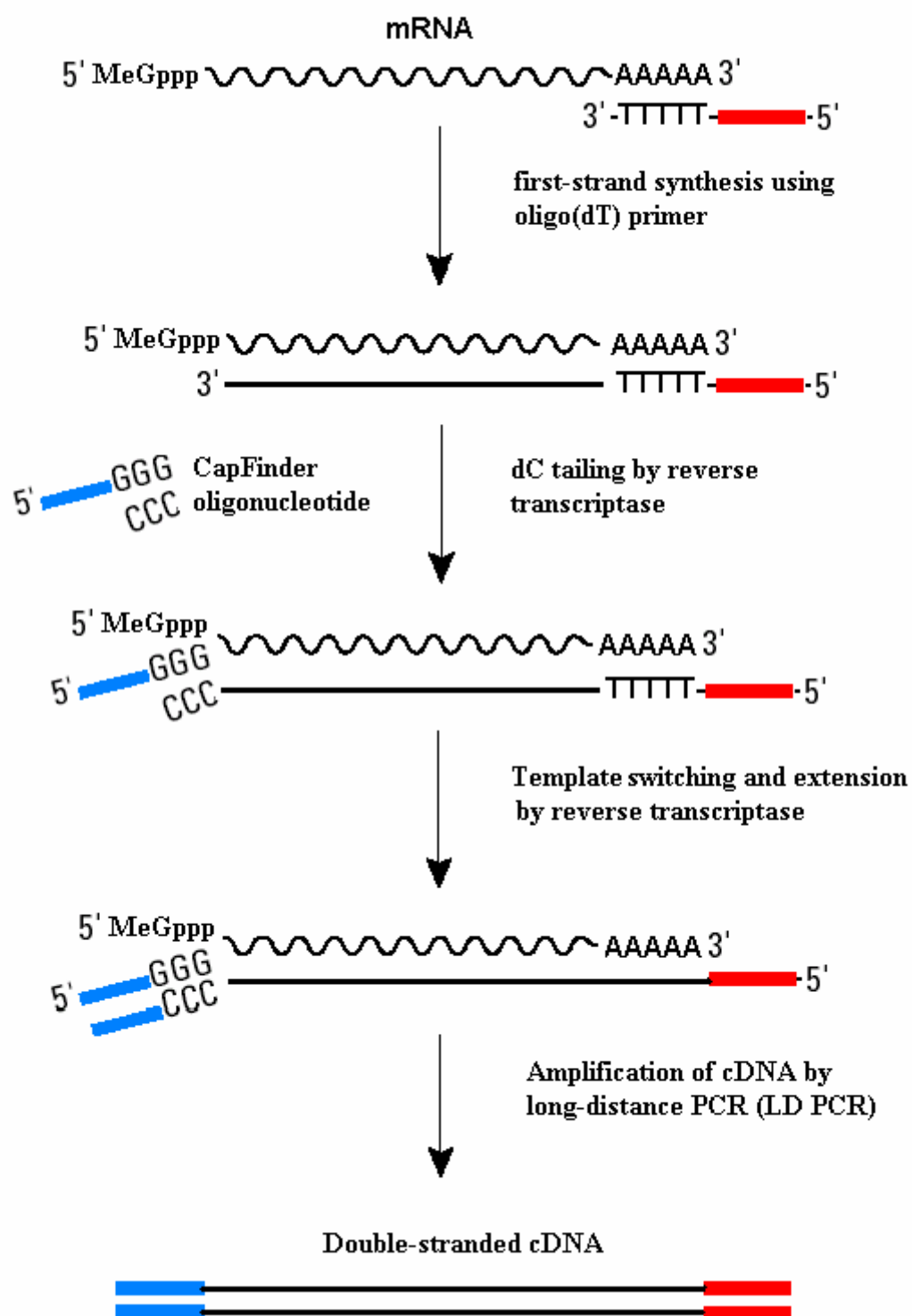
- 3) The mixture was incubated at 37°C for 5 min.
- 4) After brief centrifugation, 1  $\mu$ l of H minus M-MuLV reverse transcriptase was added and the mixture was incubated for 60 min at 42°C.
- 5) The reaction was terminated by heating at 70°C for 10 min.

- 6) The first strand product was purified using QIAquick PCR Purification Kit and eluted in 30  $\mu$ l 10 mM TrisHCl, pH 8.5.

#### 2.3.4.3 cDNA synthesis using CapFinder

##### 2.3.4.3.1 Principle

All of cDNA synthesis methods depend on the ability of reverse transcriptase to transcribe mRNA into single-stranded DNA. However, since the enzyme cannot always transcribe the entire mRNA sequence, the 5'-ends of genes tend to be under-represented in cDNA populations. This is often the case for long mRNA, especially if a oligo (dT) primer is used during the first-strand synthesis. The so called CapFinder approach offers a solution of this central problem and also allows analysis of limited starting material [Clontech Laboratories , 1996]. The method is based on the terminal transferase activity of reverse transcriptase to add cytosine residues to the 3'-end of newly synthesized cDNAs after reaching the 5'-end (cap region) of the mRNA. Usually, only 1-2 cytosine residues are added. However, it was found, that the transferase activity of reverse transcriptase is significantly improved, when the reaction included  $MnCl_2$ . In this case, 5% of the reaction products contained 4 additional cytosine [Schmidt and Mueller, 1999]. The CapFinder oligonucleotides, which has an oligo(G) sequence at its 3'-end, base pairs with this deoxycytidine stretch and creates an extended template. The reverse transcriptase then switches the template and continues replicating to the end of the CapFinder oligonucleotide. The resulting single stranded cDNA contains the 5'-end of the mRNA template, as well as a sequence complementary to the CapFinder oligonucleotide. This complementary sequence, together with the sequence of oligo(dT) primer, serves as a priming site for cDNA amplification, generating double-stranded cDNA [Figure 10]. Thus, the CapFinder method generates higher yield of full-length cDNAs than conventional methods.



**Figure 10** Overview of CapFinder cDNA synthesis [Clontech Laboratories, 1996]

#### 2.3.4.3.2 Procedure

- 1) The following components was added to a nuclease-free thin-walled PCR tube:

components	amount
total RNA	0.5-2 $\mu\text{g}$
oligo(dT) primer	10 pmoles
CapFinder oligonucleotide	10 pmoles
DEPC-treated water	to 9.1 $\mu\text{l}$
total volume	9.1 $\mu\text{l}$

- 2) To denature RNA, the mixture was incubated for 10 min at 70°C and was then chilled on ice for 1 min. After the contents was collected by brief centrifugation, the following was added in the order given:

components	volume ( $\mu\text{l}$ )
5x reaction buffer	4
10 mM dNTP mix	2
(0.1 M) DTT	2
(10 mg/ml)BSA	0.4
(100 mM) $\text{MgCl}_2$	0.6
(40 U/ $\mu\text{l}$ ) RNasin	0.5
total volume	9.5

- 3) The mixture was incubated at 37°C for 5 min.
- 4) After brief centrifugation, 1  $\mu\text{l}$  of H Minus M-MuLV Reverse Transcriptase was added and the mixture was incubated for 60 min at 42°C.
- 5) 0.4  $\mu\text{l}$  of  $\text{MnCl}_2$  (100mM) was added and the reaction was incubated at 42°C for further 15 min.

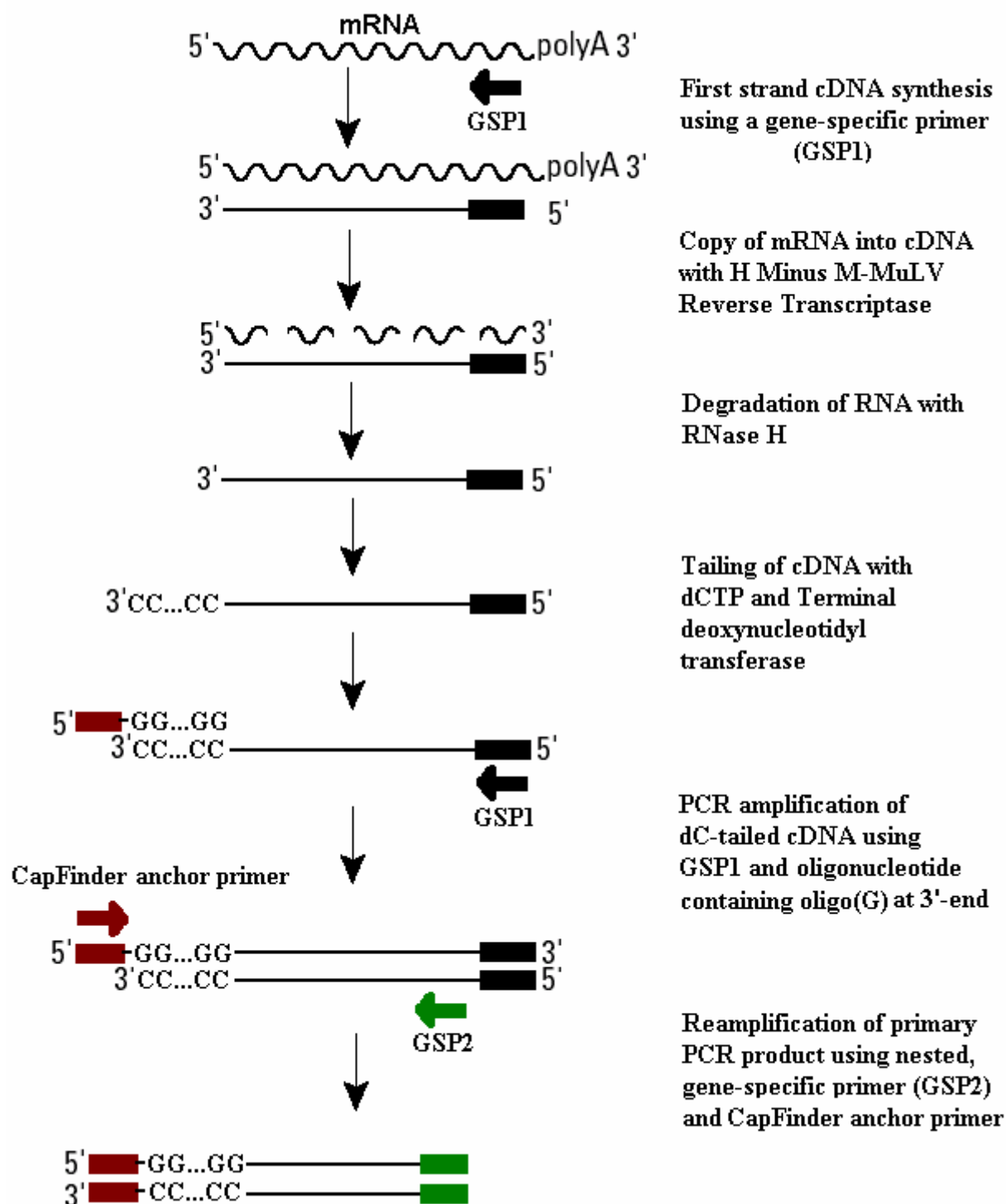
- 6) The reaction was terminated by heating at 70°C for 10 min.
- 7) The first strand product was then purified using QIAquick PCR Purification Kit.

#### 2.3.4.4 5' RACE

##### 2.3.4.4.1 Principle

Rapid Amplification of cDNA Ends (RACE) is a procedure for amplification of DNA sequence between a defined internal site and unknown site at either the 3'- or the 5'-end of the mRNA. RACE methods facilitate the isolation and characterization of both ends of the template. 5' RACE is based on tagging the 5'-ends of cDNA by means of different methods, i.e. homopolymeric tailing. The principle of 5' RACE using homopolymeric tailing is similar to those of CapFinder cDNA synthesis, except that a gene specific primer is used instead of oligo(dT) primer and CTP residues are added by the enzyme called terminal deoxynucleotidyl transferase (TdT). The main advantage of this method is that more CTP residues are added, independent from  $MnCl_2$ . As 6-8 residues are usually added, the subsequent annealing of oligo(dG) primer to the 3'-end the cDNA is significantly improved. Figure 11 shows an overview of 5' RACE: The first-strand synthesis is performed using a gene specific primer. The newly synthesized Tail cDNA is then amplified by PCR using a gene specific primer and a primer containing oligo(dG) at the 3'-end. This allows the capture of unknown sequence that lies between the gene-specific primer and the 5'-end of the mRNA. The first amplification is followed by a nested PCR, to increase the yield of specific products.





**Figure 11** Overview of 5' RACE using terminal deoxynucleotidyl transferase and dCTP

#### 2.3.4.4.2 Procedures

- 1) First-strand cDNA synthesis: The procedure was the same as using oligo(dT) primer (see 2.3.1), except that 10 pmol of gene specific primer was added instead. After the reaction was terminated by heating at 70°C for 10min, 1 µl of RNaseH was added and incubated for 30 min at 37°C to remove RNA template. The first strand product was then purified from unincorporated dNTPs and primer, using QIAquick PCR Purification Kit.
- 2) TdT Tailing of cDNA: The following components was added and gently mixed:

components	volume (µl)
sterile, distilled water	8.5
5x tailing buffer	5.0
10 mM dCTP	0.5
purified cDNA	10
final volume	24

The sample was incubated for 2 min at 95°C and chilled on ice for 1 min. After the content was collected by brief centrifugation, 1 µl Terminal deoxynucleotidyl transferase was added and the tube was incubated for 10 min at 37°C. To terminate the reaction, the TdT was heat inactivated for 10 min at 65°C. The sample was stored at –20°C

- 3) PCR of dC-tailed cDNA: PCR sample was set up by combining 5 µl of dC-tailed DNA CapDirect I, gene-specific primer and the components shown in 2.3.2.2
- 4) The same cycling parameters was used as described in 2.3.2.2 to amplify dC-tailed DNA.
- 5) Nested PCR: 5 µl of the primary PCR was diluted into 495 µl TE buffer. and 5 µl of the dilution was added to a PCR tube containing nested gene-specific primer, CapDirect anchor primer 1 and other components as

shown in 2.3.2.2. The cycling parameter of the secondary PCR was the same as the primary.

- 6) The nested PCR product was loaded to an 1.5 % agarose gel. The DNA fragment was isolated using QIAquick Purification Kit and was then cloned into a pGEM<sup>®</sup> T vector for subsequent sequencing.

## 2.4 Cloning of DNA fragments

### 2.4.1 Principle

Subcloning of a gene or a DNA fragment into an appropriate vector is an essential technique that is widely used in molecular biology. Two of the major advantages of this method are: (1) DNA fragment can be amplified up to 300-fold by plasmid replication in *E. coli*; (2) vectors used for cloning usually contains SP6, T7 or T3 promoter, which allows sequencing of the DNA insert on both directions. The general procedures start with restriction enzyme digestion on both the vector and DNA, generating sticky or blunt ends. The ligation is followed by ligation using T4 DNA ligase and transformation into an appropriate bacterial strain in which plasmids are replicated up to 300 copies per cell.

Alternatively, DNA fragment generated by a PCR can be directly ligated into a T/A cloning vector, such as pGEM<sup>®</sup>T-vector. *Taq* polymerase often adds a single deoxyadenosine to the 3'-ends of the amplified fragments. pGEM<sup>®</sup>T-vector includes a 3' terminal thymidine at both ends of the insertion site, which are compatible with the A-overhangs of the PCR products (Figure 12). Furthermore, the 3'-T overhangs of the vector improve the efficiency of ligation of the PCR product by preventing recircularization of the vector. pGEM<sup>®</sup>T-vector contain T7 and SP6 RNA polymerase promoters and the  $\alpha$ -peptide coding region of the enzyme  $\beta$ -galactosidase. Insertion of DNA fragment leads to the inactivation of this enzyme and allows recombinant clones to be identified by color screening on indicator plates. White colonies usually contain insert DNA, while blue colonies indicate the intact activity of  $\beta$ -galactosidase.

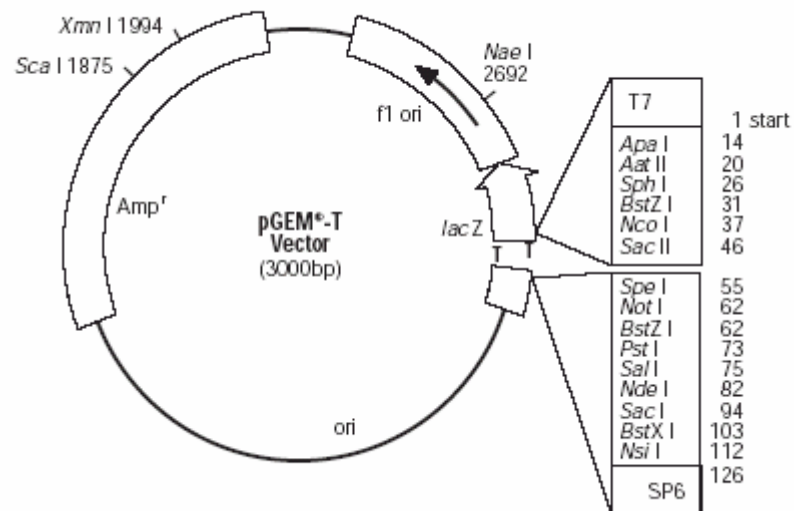


Figure 12 pGEM®T-vector

The pGEM®T-vector system has been optimized using a 1:1 molar ratio of the control insert DNA to the vector. However, ratio optimizing may be necessary in some case. Ratio of 3:1 provides a good initial parameter. The appropriate amount of insert can be calculated using the following equation:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \frac{3}{1} = \text{ng of insert}$$

## 2.4.2 Procedures

### 2.4.2.1 Ligation of DNA fragment with a pGEM<sup>®</sup>-T vector

- 1) Ligation reaction contained the following components:

components	volumes( $\mu$ l)
sterile Millipore-water	to 10
2x Rapid Ligation Buffer	5
pGEM <sup>®</sup> -T vector (50 ng)	1
PCR product	variable
T4 DNA ligase	1
final volume	10

- 2) The sample was mixed by pipetting and was incubated overnight at 4°C

### 2.4.2.2 Transformation of ligated DNA

- 1) DH10B competent cells were removed from –80°C storage and were placed on ice to thaw. The cells were then mixed by gently flicking the tube.
- 2) 50  $\mu$ l of the competent cells were transferred into the tube containing the ligation reaction. The sample was gently mixed as in step 3 and was incubated on ice for 30 min.
- 3) The competent cells were heat-shocked for 45 seconds in a water bath at 42°C and the tube was immediately placed on ice for 2 min.
- 4) 450  $\mu$ l room temperature SOC medium was added and the sample was incubated for 1 h at 37°C with shaking.

- 5) The cells were then pelleted by centrifugation at 1,000x g for 2 min and was resuspended in 200 µl SOC medium. 100 µl was plated onto two LB/ampicillin/IPTG/X-Gal plates.
- 6) The plates were incubated overnight at 37°C.

#### 2.4.2.3 Isolation of plasmid DNA

- 1) A single white colony was inoculated in 5 ml LB medium containing ampicillin and was incubated at 37°C overnight with shaking.
- 2) Plasmid DNA was isolated from the overnight culture by using Nucleospin® Plasmid Kit (see 2.2.5).

#### 2.4.2.3 Verification of DNA insertion by restriction enzyme digestion

- 1) To confirm the insertion of DNA fragment, the vector plasmid was digested at the inserting sites. Each digestion reaction included the following reagents:

components	volumes( µl)
sterile Millipore-water	13,8
NEB 2 Buffer	2
10 mg/ ml BSA	0.2 µl
plasmid DNA	2 µl
Nco I	1 µl
Spe I	1
final volume	20

- 2) After digestion of the plasmid DNA, electrophoresis was carried out on 2% agarose gel including a MassRuler as DNA standard.

- 3) Usually two bands should be observed. The upper band at 3 kb indicated the presence of the vector, while the lower band represent the insert fragment. The amount of the vector plasmid yielded was estimate by comparing the intensity of the upper band with those of the standard.
- 4) 1 µg of plasmid DNA was then vacuum dried in a SpeedVac and was sent for sequencing.

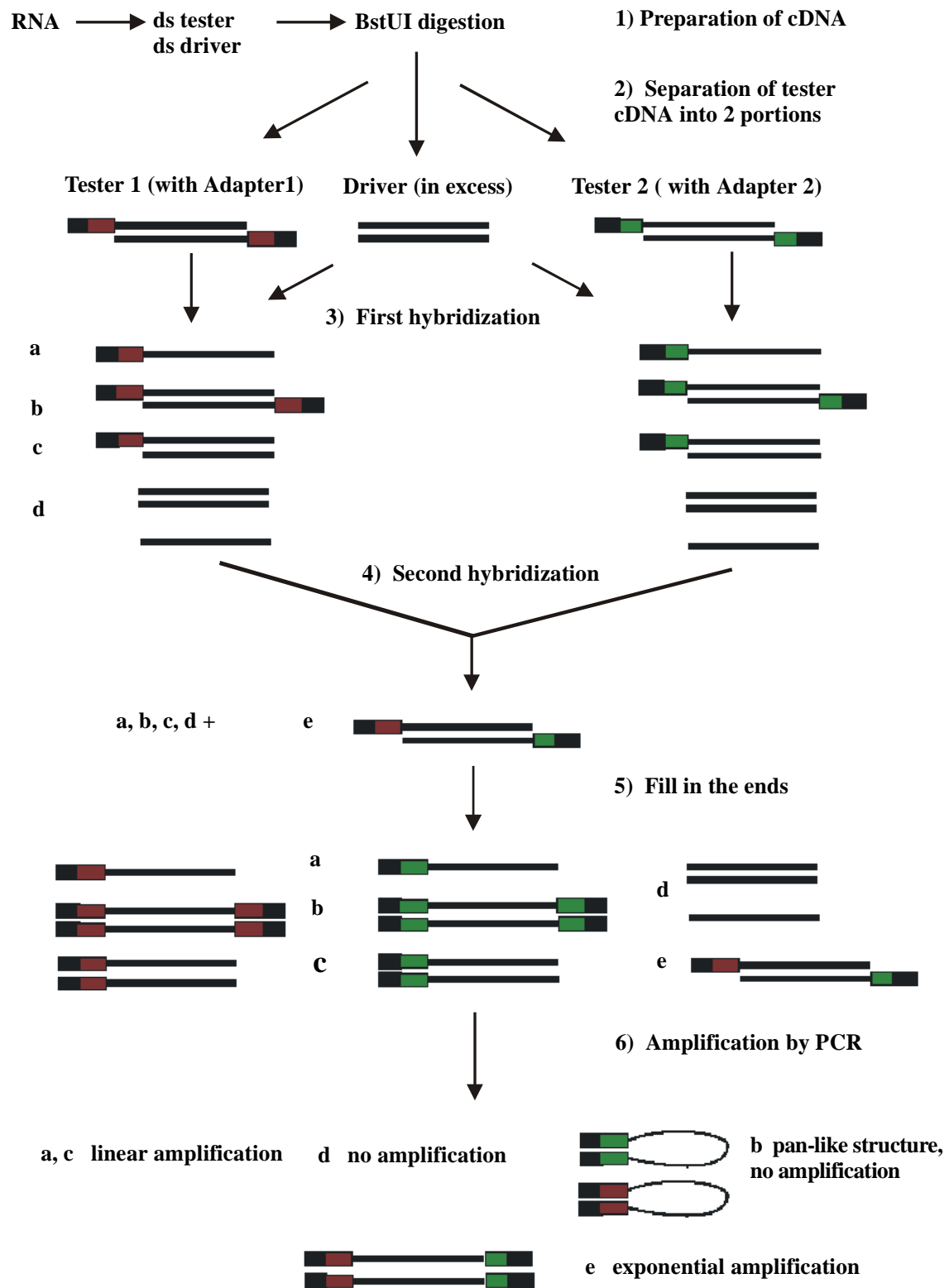
## 2.5 Suppression subtractive hybridization

### 2.5.1 Principle of suppression subtractive hybridization

Subtractive hybridization methods are usefool tools for identifying differentially regulated genes involving in cellular growth and differentiation. However many of these techniques require large amount of starting material and the yield of rarely expressed transcripts is uasualy poor. However, this minor fraction is particularly of interest, since transcripts for many regulatory proteins fall into this category. Suppression subtractive hybridization (SSH) overcomes this problem by normalizing (equalizing) sequence abundance among the target cDNA population. The normalization is based on the suppression effect of long inverted terminal repeats in polymerase chain reaction. Such inverted repeats form stable pandle-like loop structures after each annealing cycle, when attached to the ends of DNA fragments. In a PCR, the panhandle-like structures cannot be amplified exponentially, because intramolecular annealing of the long inverted repeats is more stable and therefore favored than intermolecular annealing of the shorter PCR primers. Thus, undesirable DNA fragments can be eliminated from a mixture of target sequences by integrating this effect in the subtraction scheme. The scheme of suppression subtractive hybridization is shown in Figure 13. The procedure includes six general steps. First, total RNA is isolated from cell types being compared. The RNA population contains the differentially expressed sequences, is termed as “tester”, while the “driver” population works as reference. From tester and driver mRNAs double-stranded cDNAs are synthesized and digested with a four base-cutting restriction enzyme to create blunt ends (step 1). The tester cDNA fragments are then subdivided into two

portions and ligated with two kinds of adapters. Since commercial purchased oligonucleotides using for create a double-stranded adapter, contain no phosphate groups on both ends, only the longer strand can be covalent attached to the 5'-ends of the digested cDNA (step2) (The shorter strand are melted and get loss during the first hybridization). The excess driver cDNA is mixed to a small amount of each tester cDNA. In the third step, the samples are melted and allowed to anneal, generating the type a (ss-tester), b (tester-tester), c (tester-driver) and d (driver-driver homohybrid and ss-driver). During this first hybridization step the concentration of high and low abundant cDNAs is equalized, because the reannealing process is faster for more abundant molecules. Less abundant cDNAs remain single-stranded. During the second hybridization (step 4), the two primary hybridization samples are mixed together without denaturing. Under this condition, only the remaining subtracted ss tester cDNAs can reassociate and form a new hybrid type (type e, tester1-tester2). These new hybrids contain the Adapter 1 sequence at one 5'-terminus and the Adapter 2 sequence at the other 5'-terminus. A second portion of freshly denatured driver is added to enrich the hybrid fraction e. The entire population of molecules is then subjected to PCR using adapter primers, to amplify the desires differentially expressed sequences. During the first cycle of the primary PCR, the adapter ends are filled in by DNA polymerase, creating primer binding sites (step 5). As type d (driver-driver and ss-driver) molecules do not contain primer binding sites, they cannot be amplified. Type a (ss-tester) and c (tester-driver) molecules have only one primer annealing site and can only be amplified linearly. Type b (tester-tester) molecules contain long inverted repeats on the ends and form a pan-like structure that prevent their exponential amplification (PCR suppression effect). Thus only type e molecules, which have two different adapter sequences at their ends , can be amplified exponentially. A secondary PCR amplification is performed using nested primers to reduce any background products and to further enrich differentially expressed sequences. The cDNAs can be directly inserted into a T/A cloning vector for subsequent analysis.





**Figure 13** : Schematic diagram of suppressive subtractive hybridization [according to Diatchenko et al, 1999]

## 2.5.2 Experimental procedure

### 2.5.2.1 Isolation of poly(A)<sup>+</sup> RNA

To avoid contamination with ribosomal RNA, that may results in inefficient subtractive hybridization and to enrich very rare messages, poly(A)<sup>+</sup> RNA was used for the subsequent first-strand cDNA synthesis instead of total RNA. For each tester (plasmodium) and driver (amoebae) sample, poly(A)<sup>+</sup> RNA was isolated from 25µg of total RNA using oligo(dT)<sub>25</sub> paramagnetic beads (see 2.2.2). The isolated poly(A)<sup>+</sup> RNA was eluated in 15µl 10mM Tris-HCL pH 7.5

### 2.5.2.2 First-stranded cDNA synthesis

- 1) The following components was mixed in a nuclease-free thin-walled PCR tube:

components	volume (µl)
poly(A) <sup>+</sup> RNA	5
10 µM oligo(dT) primer	1
10 µM CapFinder oligonucleotide	1
DEPC-treated water	2.1
total volume	9.1

- 2) The mixture was incubated for 10 min at 70°C and was chilled on ice. The following was added in the order given:

components	volume ( $\mu$ l)
5x reaction buffer	4
10 mM dNTP mix	2
(0.1 M) DTT	2
(10 mg/ml)BSA	0.4
(100 mM) MgCl <sub>2</sub>	0.6
(40 U/ $\mu$ l) RNasin	0.5
total volume	9.9

- 3) The mixture was incubated at 37°C for 5 min.
- 4) After brief centrifugation, 1  $\mu$ l of H Minus M-MuLV Reverse Transcriptase was added and the mixture was incubated for 60 min at 42°C.
- 5) 0.4  $\mu$ l of MnCl<sub>2</sub> (100mM) was added and the reaction was incubated at 42°C for further 15 min.
- 6) The reaction was terminated by heating at 70°C for 10 min.
- 7) The first strand product was then purified using QIAquick PCR Purification Kit
- 8) The purified ss cDNA was then used in the following long-distance PCR to create ds cDNA.

#### 2.5.2.3 Analysis of synthesized cDNA

To confirm that the first-stranded cDNA synthesis was successful, the abundance of a housekeeping gene, a rare transcript and stage-specific genes was analysed using polymerase chain reactions.

- 1) Each sample contained the following reagents:

components	amount
10x PCR buffer	5 $\mu$ l
10 mM dNTP mix	1 $\mu$ l
cDNA	24 ng
10 $\mu$ M forward primer	1 $\mu$ l
10 $\mu$ M reverse primer	1 $\mu$ l
sterillized, distilled water	to 49.5 $\mu$ l
<i>Taq</i> polymerase (5U/ $\mu$ l)	0.5 $\mu$ l
total volume	19 $\mu$ l

- 2) 30 cycles of PCR ( $T_a = 50^\circ\text{C}$  30 s) were performed.  
 3) PCR products were then analysed on an 2% agarose gel.

#### 2.5.2.4 Long-distance PCR (LD-PCR)

The LD-PCR was performed using Advantage<sup>TM</sup> 2 PCR Kit. The polymerase mix included in this kit is comprised of TITANIUM<sup>TM</sup> *Taq*DNA Polymerase, TaqStart<sup>TM</sup> Antibody to provide automatic hot start and a minor amount of a proofreading polymerase. TITANIUM *Taq* provides increase efficiency and sensitivity and is especially useful for amplifying a wide size range of DNA fragments. Targets can be amplified using fewer PCR cycles, resulting in lower background. Furthermore, the inclusion of a proofreading polymerase reduces the error rate.

For each cell type, three samples were set up: two for the subtraction hybridization and one “extra” tube to determine the optimal number of PCR cycles. Overcycled cDNA is a poor template for cDNA subtraction, while undercycling results in a lower yield. Choosing the optimal number of PCR cycles ensures that the ds cDNA remain in the exponential phase of amplification. The plateau is reached, when there is no increase in yield of PCR products with more cycles. The optimal number

of cycles is defined as one cycle fewer than is needed to reach the plateau. The LD-PCR was performed as following:

- 1) 90  $\mu$ l of TE buffer was added to the first-strand cDNA.
- 2) The following components were mixed in a PCR tube:

components	volume ( $\mu$ l)
deionized, sterile water	66
10x Advantage 2 PCR Buffer	10
10 mM dNTP	2
10 $\mu$ M oligo(dT) anchor primer (EcoRV-21)	10
10 $\mu$ M CapFinder anchor primer (CapPrimer1)	10
diluted ss cDNA	2
total volume	100

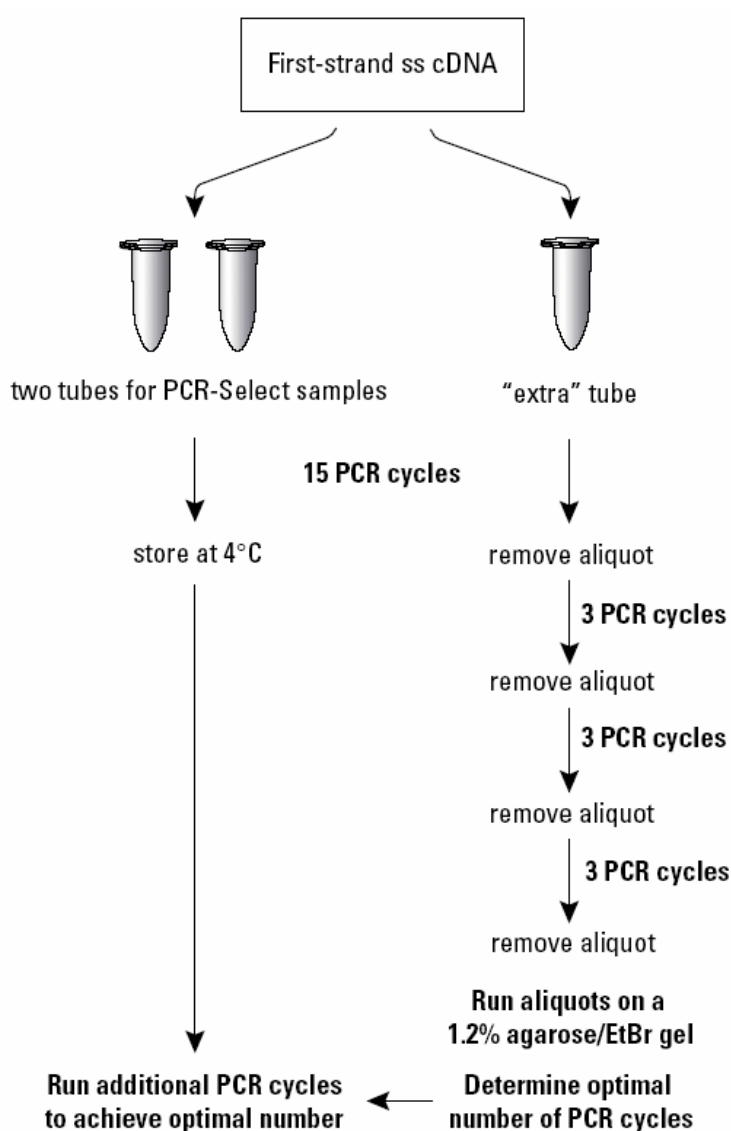
- 3) The samples were mixed by vortexing and spined briefly in a microcentrifuge.
- 4) The PCR was performed using the following cycling protocol:

initial denaturation:	95°C for 1min
cycle: 15x	
denaturation:	95°C for 30 s
annealing of primer:	55°C for 30 s
primer extension:	68°C for 3 min
final extension:	68°C for 3 min

- 5) The “extra” tube was then used to determine the optical number of PCR cycles. The other tubes were stored at 4 °C. To determine the optical cycling number (see Figure 14), 15  $\mu$ l from the “extra” tube was removed and saved for the agarose gel analysis. Three additional cycles were performed with the remaining 85  $\mu$ l of the mixture. Again, 15  $\mu$ l was

removed and three additional cycles were performed with the remaining mixture. These steps were repeated until 24 cycles was reached.

- 6) 5 µl of each aliquots from the “extra” tube was then analyzed on a 1.2 % agarose gel and the optimal cycling number was determined. The 15-cycle PCR tubes were retrieved from 4°C and additional cycles were performed, until the optimal cycling number was reached.
- 7) The PCR products were then purified using QIAquick PCR kit and the yield was estimated by UV spectrophotometry.



**Figure 14** Optimizing PCR parameters for LD-PCR [SMART cDNA Synthesis Kit User manual]

### 2.5.2.5 BstUI digestion

This step generates shorter, blunt-ended ds cDNA fragments, which are suitable for efficient subtraction. Before proceeding with BstUI digestion, 10 µl of purified ds cDNA was set aside for later analysis on a agarose gel.

- 1) As subtractive hybridization usually requires 7.5 µg driver, three samples, each contained 3 µg DNA, were set up for the digestion. In the case of tester, one sample with 300 ng DNA was sufficient for the hybridization procedure. The digestion reactions were performed by combining the components shown in Table 11.

	tester	driver
cDNA	300 ng	3 µg
10x NEB2 Buffer	5 µl	5 µl
RNase free-water	to 50 µl	to 50µl
BstUI (10U/µl)	0.5 µl	1.5 µl
Total volume	50 µl	50 µl

**Table 11** Preparation of blunt-ended cDNA

- 2) The samples were then incubated at 60°C for 1.5 h
- 3) The digested DNA was purified using QIAquick PCR Purification Kit
- 4) To confirm that the digestion was successful, 10 µl of uncut cDNA and 10 µl of digested cDNA was electrophoresed on 1.2 % agarose gel.

### 2.5.2.6 Adapter ligation

- 1) For each tester, two samples were set up containing reagents in Table 12.

	tester 1	tester 2
cDNA	100 ng	100 ng
10 $\mu$ M GWA-L1	2 $\mu$ l	
10 $\mu$ M GWA-S1	2 $\mu$ l	
10 $\mu$ M GWA-L1		2 $\mu$ l
10 $\mu$ M NKA 8 R2		2 $\mu$ l
2x Rapid Ligation Buffer	10 $\mu$ l	10 $\mu$ l
RNase free-water	2.5 $\mu$ l	2.5 $\mu$ l
Total volume	19.5 $\mu$ l	19.5 $\mu$ l

**Table 12** Preparation of master mix for the ligation analysis

- 2) The samples were heated at 50°C for 10 min. The oligonucleotides were then annealed by cooling gradually to 10°C to create ds adapters.
- 3) 0.5  $\mu$ l of T4 DNA Ligase was added to each tube. 2  $\mu$ l of tester 1 was then mixed with 2  $\mu$ l of tester 2 in a fresh tube (unsubtracted probe).
- 4) The remaining tubes were incubated at 4°C overnight.
- 5) The samples were purified using QIAquick spin columns.

### 2.5.2.7 Analysis of ligation

Since only a successful ligation ensures the efficiency of subsequent subtraction, it is important to verify that at least 25% of the cDNA have adaptors on



both ends. This experimental step is designed to amplify fragments that span the adaptor/cDNA junction of tester 1 and tester 2. A housekeeping gene is usually applied as reference. The PCR product using one gene-specific primer (derived from a reference cDNA sequence) and one adaptor primer should be about the same intensity as the PCR product using two gene-specific primers. If the band intensity differs by more than four-fold, the ligation was less than 25% complete. In this work actin was used as a reference.

- 1) Samples for PCR were prepared by combining the reagents in Tab 13. in the order shown:

	tube 1	tube 2	tube 3
10x PCR buffer with $(\text{NH}_4)_2\text{SO}_4$	5 $\mu\text{l}$	5 $\mu\text{l}$	5 $\mu\text{l}$
25 mM MgCl	4 $\mu\text{l}$	4 $\mu\text{l}$	4 $\mu\text{l}$
distilled, sterilized water	37 $\mu\text{l}$	37 $\mu\text{l}$	37 $\mu\text{l}$
unsubtracted tester (see 2.4.2.6 step3)	0.5 $\mu\text{l}$	0.5 $\mu\text{l}$	0.5 $\mu\text{l}$
10 $\mu\text{M}$ pP35 F3	1 $\mu\text{l}$	1 $\mu\text{l}$	1 $\mu\text{l}$
10 $\mu\text{M}$ pP R3	1 $\mu\text{l}$		
} actin			
Adaptor-Primer 1		1 $\mu\text{l}$	
Adaptor Primer 2			1 $\mu\text{l}$
10 mM dNTP mix	1 $\mu\text{l}$	1 $\mu\text{l}$	1 $\mu\text{l}$
<i>Taq</i> DNA Polymerase	0.5 $\mu\text{l}$	0.5 $\mu\text{l}$	0.5 $\mu\text{l}$
Total volume	50 $\mu\text{l}$	50 $\mu\text{l}$	50 $\mu\text{l}$

**Table 13** Preparation of master mix for the ligation analysis

- 2) PCR was performed using the following cycling conditions:

Extension of adaptors:	75°C 5 min
30 cycles:	
94°C	30 s
55°C	30 s
72°C	2 min
final extension:	72°C 7 min

#### 2.5.2.8 First hybridization

- 6) 3 µg of driver DNA was mixed with 100 ng of tester 1 or with 100 ng tester 2. Both samples were then ethanol precipitated by adding 1/10 volume of 3 M sodium acetate, pH 5.9 and 2.5 volume ethanol. The sample was then centrifuged at 10000 rpm and 4°C for 15 min. The pellet was washed with 70% ice-cold ethanol and the centrifugation step was repeated for 10 min.
- 7) The pellets were resuspended in each 3 µl sterile water and 1 µl 4x hybridization buffer. The solutions were overlaid with mineral oil and were denatured at 98°C for 1.5 min.
- 8) The samples were then allowed to anneal to 68°C. After 8h, the second hybridization were proceeded immediately.

#### 2.5.2.9 Second hybridization

- 1) 1 µl hybridization buffer was added to 3 µl digested driver (= 3 µg DNA). The sample was overlaid with mineral oil and was then incubated at 98°C for 1.5 min to denature driver DNA.
- 2) During this incubation, the two samples from the first hybridization were mixed together. Freshly denatured driver DNA was then immediately added and mixed by pipetting up and down. The sample was incubated at 68°C overnight.

### 2.5.2.10 Selective PCR Amplification

This step enables a selective amplification of differentially expressed cDNAs. Prior to thermal cycling, the missing strands of the adapters were filled in by a brief incubation at 75°C, thus creating binding sites for the adapter primers. In this first amplification, only ds cDNAs with different adapter sequences on each end are exponentially amplified.

- 1) The primary PCR was performed with the subtracted probe (2.5.2.9) and unsubtracted tester sample (served as a negative control for subtraction, see 2.3.3.2.5). A master mix was prepared by combine the components shown in Table 14:

components	volume (µl)
10xPCR buffer	7.5
10 mM dNTP mix	1.5
10 µM Hind III-Adapter primer	3
Bam HI-Adapter primer	3
sterilized, distilled water	52.5
Total volume	67.5

**Table 14** Preparation of a master mix

- 2) 22.5 µl of master mix was added into each of the reaction tubes, containing 2 µl of cDNA.
- 3) 0.5 µl of Advantage 2 Polymerase Mix was added to each tube. The two samples were overlaid with mineral oil and were incubated in a thermal cycler.
- 4) The mixtures were heated at 75°C for 5 min to fill the ends of the adapters, followed by 30 cycles PCR (95°C 30 s, 55°C 30 s and 68°C 3 min, the final extension was performed at 68°C for 3 min)

### 2.5.2.11 Nested PCR

To further reduce the background and to enrich for differentially expressed sequences, a second, nested PCR was performed. In the original protocol, one sample for each subtracted and unsubtracted probe were performed using nested PCR primers, which anneal to the inner part of the adapter. In this work the protocol was varied. To reduce the number of clones from one gene obtaining from the subsequent cloning procedure, two samples were setted up for each subtracted and unsubtracted probe. One tube contained CapFinder anchor primer and nested PCR annealing to adapter 1. CapFinder anchor primer 1 and nested PCR for adapter 2 were included in the other tube. Under this condition, only cDNA sequences, which contain two different adapters on the ends and CapFinder sequence could be amplified. As CapFinder oligonucleotide was used to tagg the cap region of cDNA, amplified products containing the according sequence should refer to only different mRNA species.

- 1) A master mix was prepared containing the following reagents:

components	volume (µl)
10xPCR buffer	12.5
10 mM dNTP mix	2.5
10 µM CapFinder anchor primer 1	5
sterilized, distilled water	92.5
Total volume	112.5

**Table 15** Preparation of a master mix

- 2) For each PCR reaction, the components shown in table were added in PCR tubes.

	tube 1	tube 2	tube 3	tube 4
master mix	20.5 µl	20.5 µl	20.5 µl	20.5 µl
diluted (1:10) primary PCR from subtracted sample	1 µl	1 µl		
diluted (1:10) primary PCR from unsubtracted sample			1 µl	1 µl
10 µM Nested Primer-Adaptor 1	1 µl		1 µl	
10 µM Nested Primer-Adaptor 2		1 µl		1 µl
Advantage 2 Polymerase Mix	0.6 µl	0.6 µl	0.6 µl	0.6 µl
Total volume	25 µl	25 µl	25 µl	25 µl

**Table 16** Setting up the nested PCR

- 3) 15 cycles of PCR (95°C 1 min; 15 cycles: 30 s, 53°C 30 s, 68°C 3 min, final extension 68°C 3 min) were conducted.
- 3) Tube 1 and tube 2 were then mixed together (serving as an unsubtracted probe). The mixture was purified with QIAquick PCR Purification Kit. The same procedures were performed with tube 3 and tube 4.
- 4) 8 µl from each purified sample was set aside for later analysis on an 2% agarose gel.

#### 2.5.2.12 PCR analysis of subtraction efficiency

The efficiency of subtraction can be estimated by either PCR or hybridization analysis. In both case, a nondifferentially expressed gene, e.g. a housekeeping gene is used. However, PCR provides a quicker test than hybridization analysis. In the test described below actin primers were used to confirm the reduced abundance of actin after the SSH procedure. In the subtracted sample, a PCR product should be observed about 5 to 15 cycles later than in the unsubtracted sample.

- 1) Two tubes were set up (one with the subtracted cDNA, the other with unsubtracted cDNA), each contained 24 ng DNA and the components shown in Table 17 .

components	volume (μl)
10xPCR buffer with (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub>	3
10 mM dNTP mix	0.6
10 μM pP 35 F3 (forward primer)	1.2
10 μM pP 35 R3 (reverse primer)	12
sterilized, distilled water	to 29.4
<i>Taq</i> polymerase	0.6
Total volume	30

**Table 17** Setting up the subtraction analysis

- 2) 18 cycles PCR (94°C 30 s, 54°C 30 s, 72°C 2 min) were conducted. After 18 cycles 5 μl was removed from each reaction and stored for later analysis. The samples were then transferred back to the thermal cycler for five more cycles.
- 3) Step 2 was repeated once. The 5 μl aliquots were then examined on a 2% agarose gel.

#### 2.5.2.13 Cloning and analysis of subtracted cDNAs

- 1) To analyse subtracted cDNAs, 140 ng of nested PCR products was ligated with 50 ng pGEM<sup>®</sup>-T vector. The plasmid was transformed into competent cells and was isolated and sent for sequencing (see 2.4 for detailed procedure).
- 2) The resulting sequences were compared with the Gene-Bank database using the BLAST program from NCBI.

- 3) The stage specificity of the subtracted cDNA sequences was verified by PCR analysis.

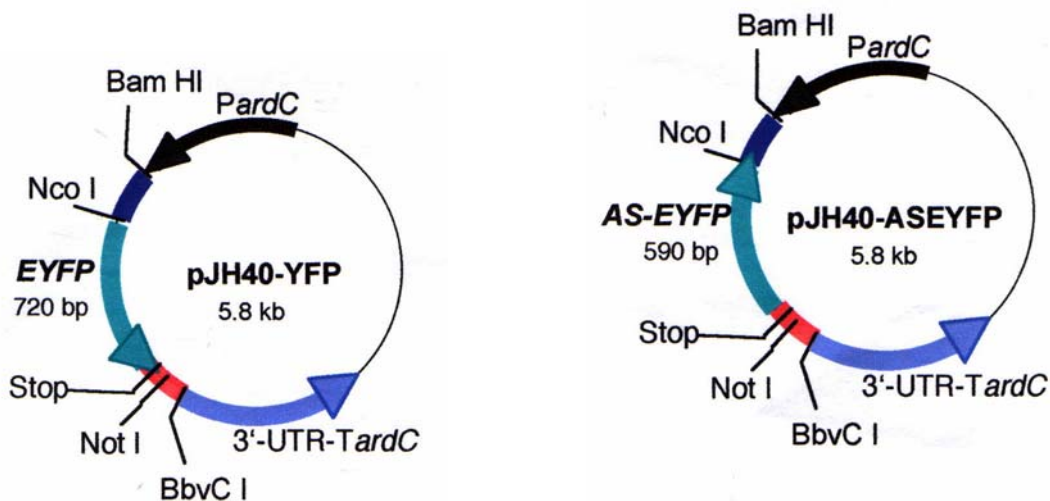
## 2.6 Knock-down assays

### 2.6.1 Antisense assays using vector encoded for enhanced yellow fluorescent protein

#### 2.6.1.1 Principle

The jelly fish, *Aequorea victoria*, emits a bluish-green light from the margin of its umbrella. The light is due to the presence of two associated proteins: aequorin (21.4 kDa) and a green fluorescent protein (GFP, 27 kDa,  $\lambda_{\text{max}} = 508 \text{ nm}$ ), which contains a hexapeptide as a chromophore. Aequorin consists of a  $\text{Ca}^{2+}$  binding apoprotein, an organic substrate and molecular oxygen. The binding of  $\text{Ca}^{2+}$  to apoaequorin triggers an intramolecular reaction in which the organic substrate coelenterazine is oxidized to coelenteramide, giving rise to a blue fluorescence protein ( $\lambda_{\text{max}} = 407 \text{ nm}$ ). An energy transfer from this blue protein to GFP generating the typical bluish-green light. Because of its easily detected green fluorescence, GFP is a widely used reporter system in studies of gene expression and protein localization. The enhanced GFP variant genes such as EGFP or EYFP contain mutations that create an open reading frame comprised preferred human codons. Furthermore, upstream sequences flanking the encoding regions have been converted to a Kozak consensus translation initiation site and potentially inhibitory flanking sequence in the original cDNA clones were removed. Furthermore, the enhanced variants fluoresce brighter than the WT and can be better quantified. Thus using of the enhanced GFP variants may enhance the translational efficiency of the mRNA and the subsequent expression. EYFP (enhanced yellow fluorescent protein) contains four amino acid substitutions that shift the emission from green to yellowish green. The fluorescence excitation maximum is 513 nm, and the emission spectrum shows a peak at 527 nm.

As the detection of GFP and its variants is easily performed, using a construct including encoded sequence for GFP or variants, was the first method of choice to analysis gene expression. To clarify whether the protein can be expressed in *Physarum*, two vectors were provided. One encodes for the EYFP (pJH40-YFP), the other one contains the anti-sense sequence of the same protein (pJH40-ASEYFP). Both vectors are derived from a pSKBluescript plasmid and contain initiation (PardC) and termination (TardC) sequence of *Physarum*-actin promoter (see Figure 15). The two vectors were transferred to the cells by microinjection. Expression of the protein was varified by fluorimetric measurement.



**Figure 15** pJH40-YFP and pJH40-ASEYFP

#### 2.6.1.2 Procedures

##### 2.6.1.2.1 Cell culture and microinjection



- 1) Both pJH40-YFP (containing the cDNA sequence of EYFP) and pJH40-ASEYFP (included antisense sequence) were subcloned into DH10B competent cells and were isolated using QIAGEN Plasmid Maxi Kit (see 2.2.6). Concentration of purified plasmid DNA was estimated using UV spectrophotometer.
- 2) For each point of the time courses three agar plates (8.5 cm) containing 300  $\mu$ l of concentrated microplasmodium suspension each plate were prepared and were incubated at 24°C for 24 h. For each time course one additional macroplasmodium plate was set up without microinjection. This plate was harvested after 24 h at 24°C and served as a negative control
- 3) Samples for microinjections were set up as described in Table 11: tube 1 contained the EYFP-sense plasmid DNA, tube 2 contained sense plasmid and antisense plasmid in excess. To rule out, that the microinjection procedure itself induce processes or substances which could affect the fluorescence, a third sample was set up with a Bluescript plasmid, which usually shows no fluorescence (tube 3).

	tube 1	tube 2	tube 3
injection buffer	10 $\mu$ l	10 $\mu$ l	80 $\mu$ l
pJH40-YFP	10 $\mu$ g	10 $\mu$ g	
pJH40-ASEYFP			50 $\mu$ g
pBluescript® II SK (+/-)		10 $\mu$ g	
sterile, distilled water	to 20 $\mu$ l	to 20 $\mu$ l	to 20 $\mu$ l

**Table 18:** Preparation of microinjections

- 3) The samples were separately injected into the three plates of macroplasmodium and the plates were returned to 24°C until they were harvested.

**2.6.1.2.2 Fluorimetric measurement**

- 1) For each time course, injected macroplasmodia were harvested at the following different points: 2.5 h, 5h, 9h, 12h, 16 h, 24 h and 27 h after microinjections.
- 2) Total cell lysis was performed as described in 2.8.1
- 3) 100 µl of the clarified lysate was added to a quartz cuvette and was set up to 1 ml with total cell lysis buffer.
- 4) The measurements were performed in a fluorescence spectrophotometer at 4°C. The emission was measured at two different excitation wavelengths (488 nm and 513 nm). For reliable quantification and measurement of light scatter, excitation was also measured by emission wavelength of 513 nm.

**2.6.1.2.3 Western Blotting**

- 1) The amount of proteins in cell extract was quantified using the Bradford assay (see 2.8.2).
- 2) SDS-PAGE was performed with 100 and 500 µg protein of each sample (see 2.8.3 for details).
- 3) Western Blotting was carried out as described in 2.8.4 using 1:400 diluted BD Living colors A.v. Peptide Antibody and peroxidase conjugated anti-rabbit Ig G (1:2500 diluted) as the secondary antibody.

**2.6.2 RNAi assays****2.6.2.1 Principle**

In animals, double-stranded RNA specifically silences expression of a corresponding gene, a phenomenon termed RNA interference. One function of the RNAi machinery is probably to maintain the integrity of the genome by suppressing the mobilization of transposons and the accumulation of repetitive DNA [Jensen et al., 1999]. The RNAi machinery may also defend cells against viral infection and regulate expression of cellular genes [Montgomery and Fire, 1998; Jensen et al., 1999]. Most of gene-silencing phenomena including co-suppression in plants, quelling in fungi and RNA interference, occur in the cytoplasm at a post-transcriptional level (PTGS) with the mRNAs of the target gene degraded in a specific manner. Small non-coding RNA molecules have been demonstrated to be mediators of these silencing phenomena and were termed small interfering RNAs [Elbashir et al., 2001]. [Smardon et al., 2000]. Further, dsRNA may also operate as a regulator of gene expression at a transcriptional level via RNA-directed DNA methylation, since methylation of promoter sequences leads to transcriptional gene silencing [Sijen et al., 2001]. dsRNA may trigger the gene silencing by two different but overlapping pathways. Both pathways begin with the degradation of dsRNA by the Dicer complex, which includes Dicer RNaseIII enzyme itself, RNA helicase and probably adapter proteins, generating small interfering RNAs (siRNA). siRNA with phosphorylated 5' termini are recognized and incorporated into the RNA-induced silencing complex (RISC), which unwinds the double-stranded siRNAs. The antisense strand of the unwound siRNAs can pair with the target mRNAs resulting in double-stranded RNAs which are then also cut by an RNA endonuclease. Alternatively siRNAs are recognized by RNA-dependent RNA polymerase (RdRP), which use the antisense strand as primer. The complementary target mRNA can base pair with the antisense strand and are extended by the polymerase. The Dicer complex then fragments the newly synthesized dsRNA.

Synthetic dsRNA can be easily generated by performing an so called *in vitro* transcription. The method relies on the ability of DNA-dependent RNA polymerases to transcribe DNA templates into single-stranded RNAs, which are then allowed to anneal, resulting in dsRNAs. The relevant DNA template can be generated either by cloning the fragment into a plasmid that contains a promoter for RNA polymerase or by amplification in a PCR using gene-specific primers whose 5'-ends encode promoters for RNA polymerase. The main advantages of the PCR method are (1)

there is no need for cloning and preparation of plasmids and (2) the probes of high specificity and of any size can be used. RNA polymerase encoded by bacteriophages T3 and T7 transcribe DNA template including the appropriate promoters with high specificity. However the RNA polymerase encoded by SP6 is reported to transcribe PCR-amplified DNA much less efficiently than linearized plasmid DNAs [Logel et al. 1992]. For this reason, using primers that encode promoters for bacteriophages T3 and T7 are recommended.

- 1) First strand cDNA synthesis from plasmidium was carried out using oligo(dT) primer (see 2.3.4.2)
- 2) To generate DNA templates for the *in vitro* transcription, PCRs were performed using gene-specific primers encode promoter for T7 RNA polymerase.
- 3) PCRs were carried out as shown in 2.3.2.2 including 2 µl of cDNA (step 1)
- 4) PCR products were then analyzed on a 2% agarose gel and the appropriate bands were isolated.
- 5) T7-RNA transcription was performed in a 50 µl reaction mix containing the following components:

components	amount
DEPC-water	to 50 µl
5x transcription buffer	10 µl
RNase inhibitor	40U
rNTPs	1 mM
purified PCR product	29 µl
yeast pyrophosphatase	0.1 U
T7-RNA polymerase	100 U
final volume	50 µl

- 6) The sample was incubated at 37°C overnight.
- 7) To terminate the reaction, 2 U of DNase I was added per 1 µg DNA template and the sample was incubated at 37°C for 30 min.

- 8) The probe was incubated at 95°C for 5 min and was then allowed to slowly cool down to the room temperature.
- 9) The annealed dsRNAs were precipitated by adding 1/10 volume of 3 M sodium acetate, pH 5.9 and 2.5 volume ethanol. The sample was then centrifuged at 10000 rpm and 4°C for 15 min. The pellet was washed with 70% ice-cold ethanol and the centrifugation step was repeated for 10 min. The pellet was air-dried and was resuspended in DEPC-water.
- 10) dsRNA was quantified in a UV spectrophotometer using the same converting value as in case of ssRNA. The integrity was verified by agarose gel electrophoresis.

## **2.8 Analysis of proteins by SDS-PAGE and Western Blotting**

### **2.8.1 Preparation of crude extract of macroplasmidium**

- 1) After the macroplasmidium was harvested, weighed and transferred into a glass homogenizer, 2 volumes of lysis buffer, 1/25 volume (as calculated from volume of lysis buffer) of protease inhibitor cocktail and 1/1000 volume (as calculated from total volume) of Mercaptoethanol were added.
- 2) After the cells were well homogenized, the sample was transferred into a clean tube and was centrifuged at 17000 rpm for 30 min to clarify the lysate.
- 3) The supernatant was carefully removed without disturbing the pellet and was centrifuged again for 15 min.
- 4) The lysate was either immediately used for analysis or was frozen in liquid nitrogen and stored at -80°C for later use.

### **2.8.2 Determination of protein concentration using the Bradford assay**

- 1) 2, 5, 7, 10, 15, 20 and 50  $\mu\text{l}$  of BSA stock solution (0.1 mg/ml) was added to the cuvettes used for the standard curve.
- 2) An appropriate volume of millipore  $\text{H}_2\text{O}$  was added to each cuvette from step 1, to give total volume of 0.1 ml.
- 3) A 1:5 dilution from the Bradford stock solution was performed with millipore  $\text{H}_2\text{O}$ . 0.9 ml of the diluted Bradford stock solution was added to every cuvette (from step 1), producing a total volume of 1 ml.
- 4) 1  $\mu\text{l}$  of crude extract was added to a cuvette and made up to 0.1 ml with millipore  $\text{H}_2\text{O}$ . 0.9 ml of the diluted Bradford reagent solution was then added.
- 5) All the samples were incubated at room temperature for 20 min. Protein measurement was then carried out at a wavelength of 595 nm in a UV-Vis spectrophotometer.

### 2.8.3 SDS-PAGE

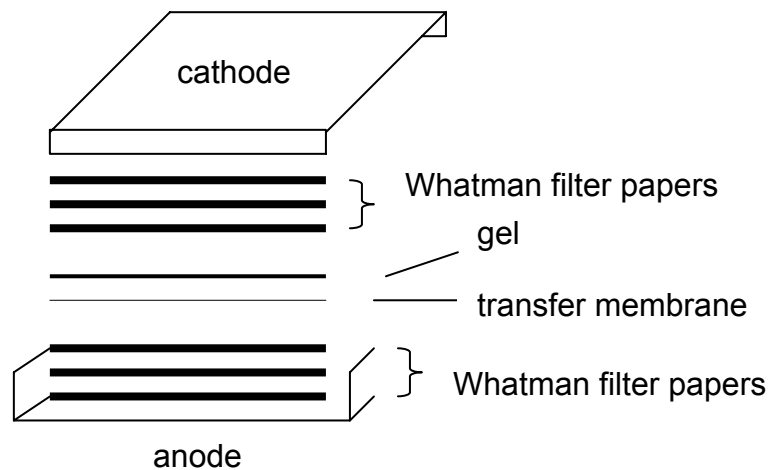
- 1) The glass plates and spacers were thoroughly cleaned with detergent and dd $\text{H}_2\text{O}$ . The air dried glass plates and spacer were wiped using paper prewetted with 100% ethanol.
- 2) The spacers were transferred between the two glass plates and fix with clamps, forming a sandwich.
- 3) The separation gel mixture was prepared with ammonium sulfate and TEMED adding at the end. The mixture was briefly vortex and immediately filled into the sandwich up to about 4 cm from the top edge.
- 4) About 0.5 ml of isopropanol was immediately loaded to make the surface of the acrylamide gel mixture even. The gel was allowed to polymerize for about 20 min. A sharp gel-alcohol interface should be seen once the gel has polymerized.
- 5) The isopropanol layer was then drained away by inverting the casting unit.
- 6) The stacking gel was then prepared, mixed and added into the sandwich. The comb was then carefully inserted into the sandwich.
- 7) While the stacking gel was polymerizing, the protein samples and the standard marker were prepared. To completely denature proteins, 1

volume of 2x sample loading buffer was added to each sample and standard marker. The samples were then incubated at 95°C for 10 min.

- 8) After the stacking gel was polymerized the comb was carefully pulled from the gel and the sandwich was tightly casted into the electrophoresis apparatus and the chambers were filled with 1x running buffer.
- 9) The wells and the bottom of the sandwich were rinsed well with the running buffer to remove any air bubbles.
- 10) The samples together with the standard marker were then loaded. The apparatus was connected to a power supply. First 10 mA was applied for one gel (1.5 mm-thick standard sized gel) until a sharp line was observed. The power was then turned on to 25 mA.
- 11) The gel was allowed to run until the dye reached about 1 cm from the bottom of the gel.

#### 2.8.4 Western Blotting

- 1) A piece of polyvinylidene difluoride (PVDF) and 6 sheets of Whatman filter paper to the same size as the gel were cut. The Whatman filter papers were soaked in transfer buffer for 5 min prior to blotting. The PVDF membrane was soaked in methanol for 15 s and later in distilled water for 2 min, prior to soaking in transfer buffer.
- 2) A blotting system was assembled in the order shown in Figure 16:  
3 sheets of the soaked Whatman papers were transferred to the plate. Any bubbles were removed by rolling a pipette over the papers. The PVDF membrane was then placed on the top of the filter papers the membrane was carefully overlaid with the gel. The remaining 3 sheets of filter papers were placed on the gel. The top plate was placed on top of the assembled unit.
- 3) A bottle containing 1 liter liquid was placed on the top of the plate to serve as a weight.
- 4) The assembled unit was connected to a power supply. Blotting was allowed to proceed for 1 h at 2 mA/cm<sup>2</sup> of the membrane.



**Figure 16** Diagram of semidry electroblotting

- 5) The membrane was then strained with Ponceau solution for 2-3 min. The membrane was washed with a small amount of distilled water and shaken until the standard marker was visible.

The membrane was washed two times with 1xPBS/Tween buffer and one time with 1x PBS buffer for 10 min each wash..

- 7) 20 ml of blocking buffer was added and the membrane was incubated overnight at 4°C.
- 8) The membrane was washed as in step 6 and incubated with the primary antibody (diluted in blocking buffer) for 2 h at room temperature with shaking.
- 9) After washing as in step 6, the membrane was incubated with diluted secondary antibody (conjugated with horseradish peroxidase) for 1 h at room temperature with shaking.
- 10) The membrane was then washed four times with PBS/Tween buffer and one time with PBS buffer for 10 min each wash.
- 11) The proteins were detected by a chemiluminescent reaction of peroxidase-conjugated antibody. For this purpose, the membrane was placed on a tissue paper. 1.5 ml of each Nova A and Nova B solution was mixed. The membrane was overlaid with the mixture. After incubation at room temperature for 1 min, the membrane was overlaid with a plastic foil and was transferred into an exposure cassette.

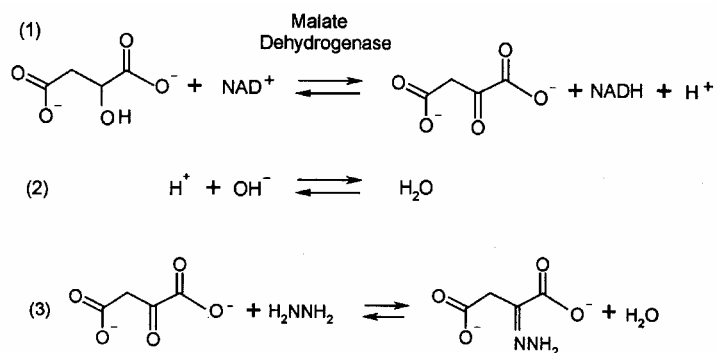


- 12) In a darkroom, a chemoluminescent detection film was placed on the plastic foil and the cassette was closed immediately. The exposure was allowed to proceed first for 5 s, to reduce any background.
- 13) The film was placed into an automatic developer. Step 12 was repeated again with increasing the exposure time to 10 s.

## 2.9 Quantitative analysis of PMLA

### 2.9.1 Principle

The amount of polymalate can be determined by different kinds of assay. The polymer can be quantified both with and without cleavage. Pure polymalate in solution, e.g., shows an absorbance at 200-230 nm and can be directly quantified using a UV spectrophotometer. However, for reliable quantification, methods of assaying after cleavage are preferable. In this case, PMLA is cleaved by sulfuric acid into L-malic acid. Free endogenous L-malate contained in the sample before hydrolysis is measured separately and subtracted from the total malate content. The amount of fumaric acid as side product of the cleavage is less than 1% and can be ignored. L-malate is then quantitatively measured by spectrophotometric reading of NADH formed by the oxidation of L-malate to oxalacetate catalyzed by malate dehydrogenase (Figure 17, step1). The reaction mixture must have a pH of 9 and must contain hydrazine to shift the equilibrium towards quantitative oxidation of L-malate (Figure 17, step2 and 3). Malate dehydrogenase is highly specific for L-malate, even in complex mixtures such as cellular extracts. NADH can be quantified either in a UV spectrophotometer or in a fluorescence spectrophotometer, whereas the latter provides more sensitivity.



**Figure 17** Assay for polymalate by enzymatic oxidation of L-malate after cleavage  
[Holler and Lee, 2002]

## 2.9.2 Procedures

- 1) Total cell lysis from macroplasmidium was prepared as described in 2.8.1
- 2) 100  $\mu\text{l}$  of the clarified lysate was removed and aliquoted equally into two microcentrifuge tubes and placed on ice. The remaining suspension was immediately freezed in liquid nitrogen.
- 3) One of the two aliquots containing 50  $\mu\text{l}$  cell extract was hydrolyzed with 50  $\mu\text{l}$  of sulfuric acid and incubated at 95°C for 1.5 h. The other tube was kept on ice and was later used to determine the amount of endogenous malate.
- 4) After 1.5 h at 95°C, 50  $\mu\text{l}$  of 4 M NaOH was added to neutralized the mixture and pH was checked with an indicator paper.
- 5) 5  $\mu\text{l}$  of both tubes were then subjected to the dehydrogenase assay.
- 6) The dehydrogenase assay was performed at 37°C in a fluorimeter with  $E_x = 340 \text{ nm}$  and  $E_m = 455 \text{ nm}$ . The assay was conducted by adding 5  $\mu\text{l}$  of the sample to a quartz cuvette containing 800  $\mu\text{l}$  of prewarmed (at 37°C) glycine-hydrazine buffer and 80  $\mu\text{l}$   $\text{NAD}^+$ . The cuvette was placed into the fluorescence spectrometer and was equilibrated until the level of fluorescence intensity was constant. 10  $\mu\text{l}$  of dliluted malate dehydrogenase was then added and briefly mixed. After no more increase

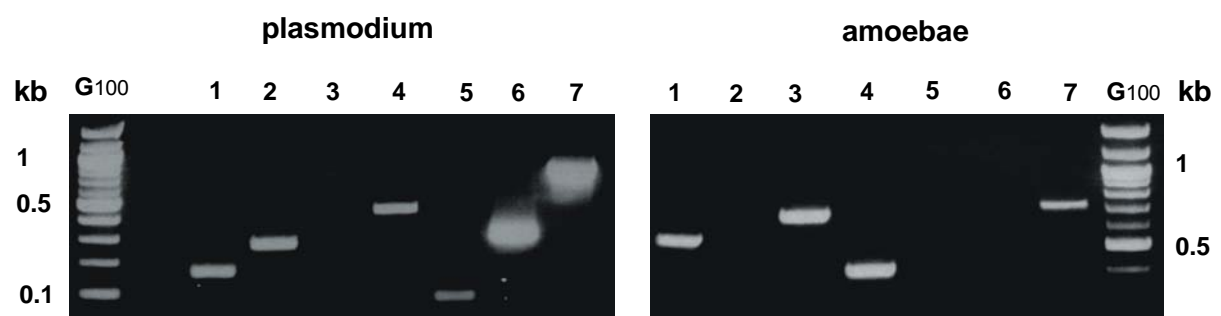
in the absorption was visible, 10  $\mu$ l of 0.1 mg/ml L-malate was added for standardization.

### III Results

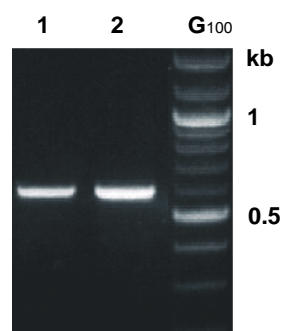
## 1 Suppression subtractive hybridization

### 1.1 Analysis of cDNA

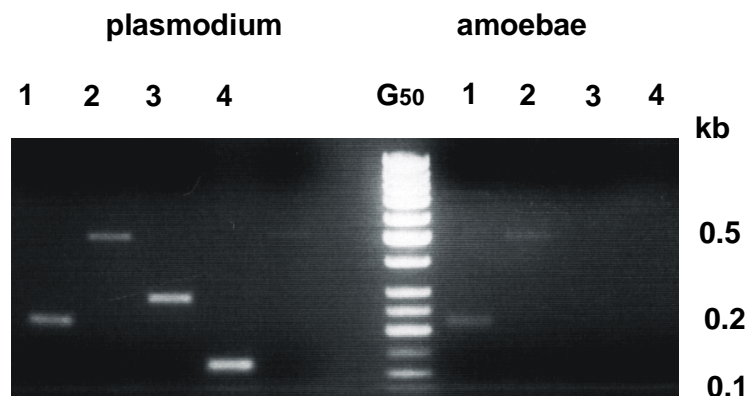
To find reference genes for the later subtractive hybridization, as well as to confirm that first-strand cDNA synthesis was succesful, the abundance of different genes, including actin, stage-specific genes and polymalatase was verified by PCR analysis. The results are shown in Figure 18-20



**Figure 18** PCR analysis of cDNA from plasmodium and amoebae Lane 1: actin. Lane 2: actin-fragmin-kinase. Lane 3: fragmin A. Lane 4: ABP-46. Lane 5: Fragmin P. Lane 6: plasmin C. Lane 7: polymalatase. G<sub>100</sub>: GeneRuler™ 100 bp.



**Figure 19:** PCR analysis of lig 1. Lane 1: cDNA from plasmodium. Lane 2: cDNA from amoebae. G<sub>100</sub>: GeneRuler™ 100 bp.

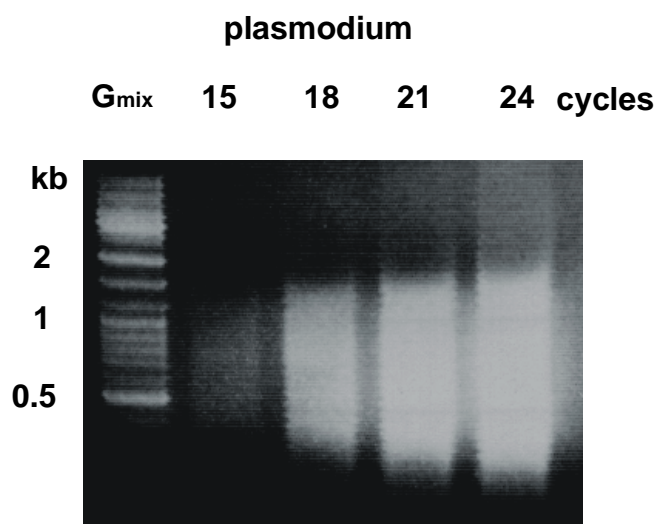


**Figure 20** Analysis of spherulin gene products in plasmodium and amoebae. Lane 1: Spherulin 1a. Lane 2: Spherulin 1b. Lane 3: Spherulin 2a. Lane 4: Spherulin 2b. G<sub>50</sub>: GeneRuler™ 50 bp.

As expected, actin as a housekeeping gene was detected with similar intensity in both cDNA populations, indicating that its expression level is kept constant during the life cycle. However, the quality of synthesized cDNA should not only be determined by the presence of one of the housekeeping genes, since they are highly abundant. A “good-working” cDNA synthesis should be able to produce rare transcripts with the same efficiency. Therefore, the abundance of *lig 1*, an early gene of the phytochrome-controlled sporulation, was verified as well. Since *lig1* is only expressed under certain conditions [Kroneder et al., 1999], it falls into the category of rare transcripts. As shown in Figure 19, a distinct band with similar intensity could be observed in both amoebae and plasmodium, indicating that cDNA synthesis was worked well in both cases. As expected, actin-fragmin kinase, fragmin P and plasmin C, which have been reported to be plasmodial-specific [Jampens et al., 1997; Jampens et al., 1999; St. Girard et al., 1990] were indeed only found in plasmodium. On the other way round, fragmin A was detected in amoebae but not in plasmodium, supporting its stage-specificity. Surprisingly, ABP-46, an amoebal specific gene, was found in both cell types. With regard to the members of the spherulin gene family, spherulin 2a and 2b are probably plasmodial specific, since they were found in plasmodium but not in amoebae. Hence, at least one of the cited plasmodial specific genes should remain after subtractive hybridization. In the other way round, commonly expressed transcripts such as actin should not be detected after subtractive hybridization.

## 1.2 LD-PCR

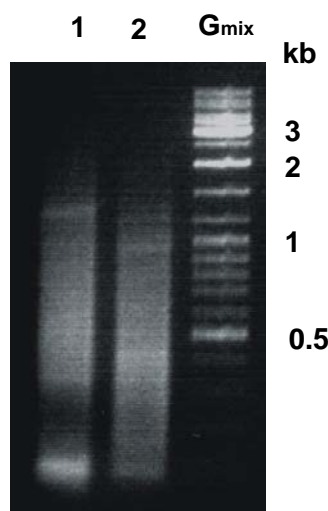
One of the disadvantages of subtractive hybridization methods is that a large amount of starting material is usually required. CapFinder cDNA sythesis including long-distance PCR provides an uncomplicated method for producing cDNA from nanograms total or poly A<sup>+</sup> RNA. However overcycled cDNA is a poor template for cDNA. Undercycling, on the other hand, results in a lower yield. Hence, it is recommended to determine the optimal number of PCR cyles to ensure that ds cDNA will remain in the exponential phase. The optimal number of cycling is characterized as one cycle lower than is needed to reach the plateau.



**Figure 20** Analysis for optimizing PCR parameters

LD-PCR with cDNA from amoebae and plasmodium showed similar pattern. In both case, the optimal cycling number was 17, since the yield of products stoped increasing after 18 cycles. A typical gel profile obtained from LD-PCRs with both cell types is shown in Figure 20. The plateau was reached after 18 cycles and a smear appeared in the higher-molecular-weigh regions after 21 and 24 cycles, indicating that the reactions were overcycled.

Thus, LD-PCRs were performed for 17 cycles and 10  $\mu$ l of each sample were loaded on a 1.2% agarose gel to compare the size distribution of the amplified products (Figure 22).

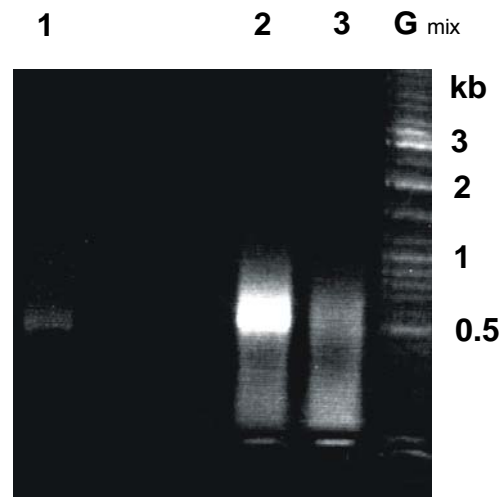


**Figure 22** LD-PCR. Lane1: amoebal cDNA population. Lane 2: plasmodial cDNA population. G<sub>mix</sub>: GeneRuler™ DNA Ladder mix.

In both case, LD-PCR was successful to amplify cDNA up to about 1.5 kb. However, size distribution for amoebae was a little smaller, since a stronger smear at the region beyond 0.3 kb was observed.

### 1.3 Analysis of ligation

Since a successful ligation ensures the efficiency of subtraction procedure, it is recommended to verify that at 25% of the cDNAs have adaptor on both ends, The ligation efficiency is about 25%, when PCR product using one gene specific primer and one adapter primer show the same intensity as the PCR product amplified using two gene-specific primers. If the band intensity differs by more than four-fold, the ligation was less than 25% complete. In this case, subtraction efficiency will be significantly reduced.



**Figure 23** Analysis of ligation efficiency. Lane 1: PCR product using two actin-specific primers. Lane 2: PCR product using actin-specific forward primer and Adaptor-Primer 2. Lane 3: PCR product using actin-specific forward primer and Adaptor-Primer 1

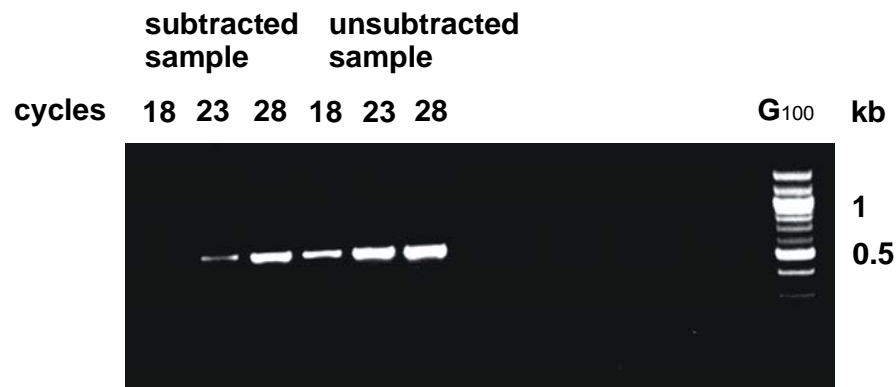
As shown in Figure 23, PCR products using an actin-specific primer and one of the adaptor primers were more than four-fold stronger than PCR product using two actin-specific primers, indicating that the ligation efficiency was more than 25%.

## 1.4 Analysis of subtraction efficiency

The central problem of subtraction procedures is the disproportion in concentrations among different types of transcripts. This usually makes the isolation of specific rare transcripts extremely difficult. However, this minor fraction is particularly of interest, since many differentially expressed transcripts fall into this category. To enrich for such rare transcripts, a satisfactory equalization during the subtractive procedure is required.

To determine the efficiency of the subtractive hybridization, the abundance of actin as one of the house-keeping genes was compared before and after the subtraction procedure. As shown in Figure 24, a PCR product was visible in the unsubtracted cDNA probe after 18 cycles. In the subtracted sample, the product was observed 5 cycles later, indicating that the subtraction procedure was successful.

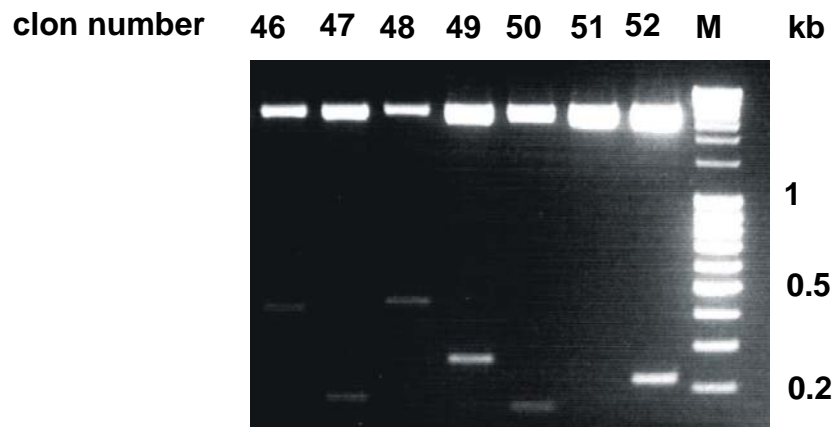




**Figure 24** Analysis of actin abundance before and after suppression subtractive hybridization.

## 1.5 Analysis of subtracted cDNAs

After suppression subtractive hybridization, differentially expressed cDNA were selected by two rounds of PCR. During the primary PCR, only cDNAs, which contained different adaptors on each ends could be exponentially amplified. To reduce the number of bacteria clones obtained from the subsequent transformation procedure, nested PCRs were performed. During these secondary PCRs select only cDNAs which contain one of the adaptors and the CapFinder sequence were selected. The amplified products were ligated with pGEM<sup>®</sup>-T vector and were transformed into competent cells. About 70 white colonies were obtained in total after transformation, 52 of which were selected. Plasmid DNAs were then isolated and analysed by restriction enzyme digestion. One of the results from agarose gel electrophoresis is shown in Figure 25



**Figure 25** Analysis of plasmid preparations. M: MassRuler™

19 of plasmid preparations containing inserts >150 bp were sent for sequencing. Tab. 19 shows an overview of obtained sequences and their characterization (only the match with the highest expect value was given).

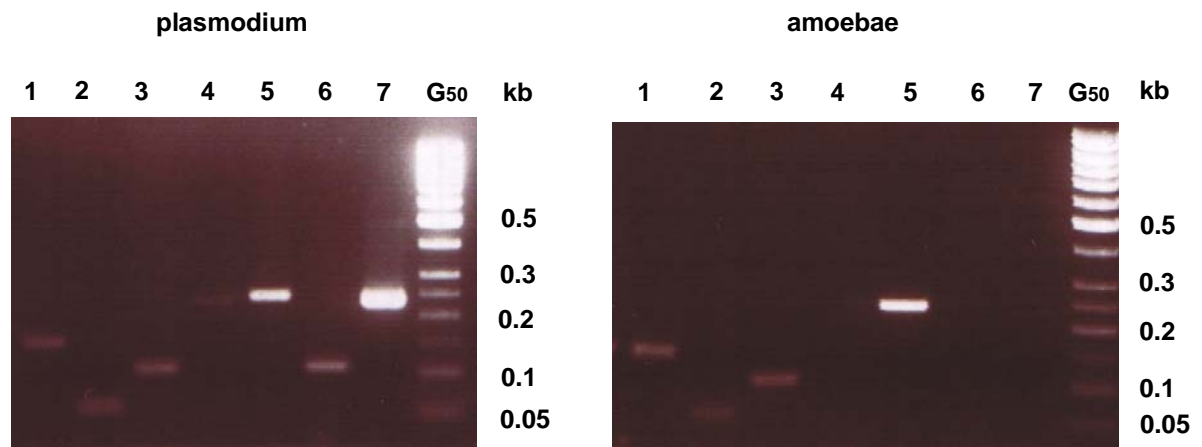
Name of cDNAs	Length (bp)	Abundance of poly A tails	possible start codons/ 5'3' ORFs	Match in Genebank (identity%/ expect)
NKA2	288	yes	0/0	no
NKA8	329	yes	0/1	no
NKA11	314	no	3/1	no
NKA12	105	yes	0/1	<i>X.laevis</i> , elongation factor 1β (90%/ 5e-11)
NKA13	129	yes	0/0	no
NKA14	142	yes	1/1	no
NKA15	180	yes	0/0	no
NKA16	167	no	4/2	no
NKA19	78	yes	2/1	no

NKA20	90	no	0/1	no
NKA22	80	yes	1/2	no
NKA23	86	yes	2/1	no
NKA27	75	yes	1/3	no
NKA35	135	yes	0/0	no
NKA37	86	no	1/1	no
NKA43	435	yes	0/0	<i>N. tabacum</i> , DNA-directed RNA polymerase IIb (86%/ 1e-05)
NKA48	335	yes	0/0	<i>P. polycephalum</i> , Spherulin 2b (88%/ 9e-62)
NKA49	165	yes	0/1	no
NKA52	115	yes	0/0	no

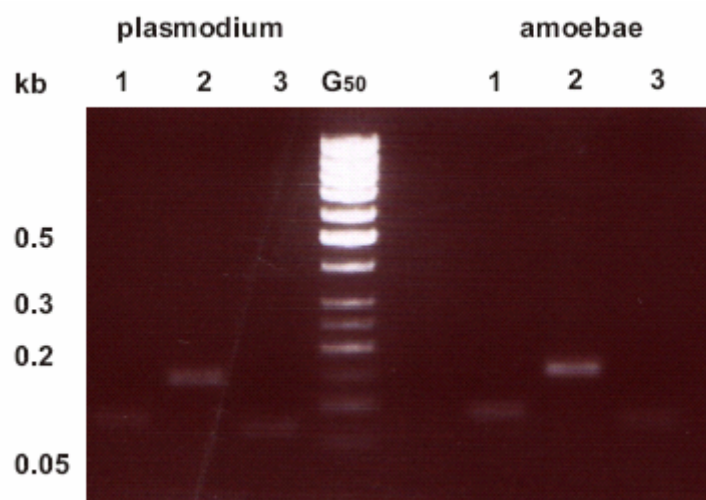
**Table 19** Characterization of sequenced cDNAs (bp = base pair, ORF = open reading frame, i.e., when no stop codon is included)

10 of the subtracted cDNAs showed appropriate length to be efficiently amplified by PCRs. Thus, the plasmodial specificity of these 10 cDNAs was verified by PCR analysis. The results are shown in Figure 26 and 27.

The results indicated that only NKA8, NKA48 and NKA49 are plasmodial-specific, since they could not be detected in amoebae. In the case of NKA8, only trace amount of the PCR product was observed, suggested that it may be fall into the category of very rare transcripts. In contrary, PCR analysis of NKA48 indicated high abundance.

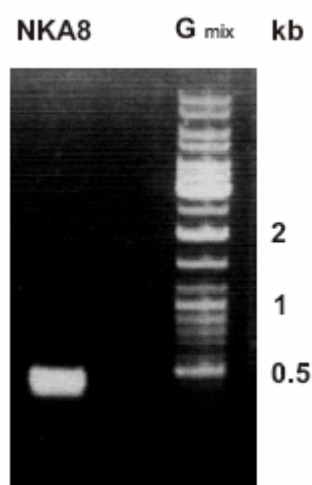


**Figure 26** PCR analysis of subtracted cDNAs, I. Lane 1: NKA11 (F1/R1). Lane 2: NKA 14 (F1/R1). Lane 3: NKA2 (F1/R1). Lane 4: NKA8 (F2/R2). Lane 5: NKA43 (F1/R1). Lane 6: NKA 49 (F1/R1). Lane 7: NKA48 (F1/R1).

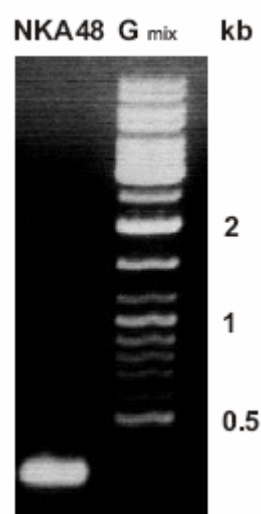


**Figure 27** PCR analysis of subtracted cDNA sequences, II. Lane 1: NKA15 (F1/R1). Lane 2: NKA 16 (F1/R1). Lane 3: NKA20 (F1/R1).

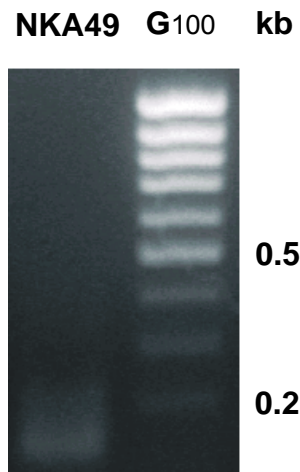
No possible start codon (ATG) was found in the differentially expressed cDNAs (NKA8, NKA 48 and NKA49), suggested that the cDNAs obtained were not complete. To obtain the missing 5'-region of these cDNAs, 5' RACE was performed using one gene-specific primer and the CapFinder oligonucleotide. The results are shown in Figure 28-29.



**Figure 28** Nested PCR from 5'-RACE of NKA8 (R3/CapFinder anchor primer)



**Figure 29** Nested PCR from 5'-RACE of NKA48 (R2/CapFinder anchor primer)



**Figure 30** Nested PCR from 5'-RACE of NKA49 (R2/CapFinder anchor primer)

The amplified products from 5'-RACE were ligated again with pGEM<sup>®</sup>-T vector. After transformation, plasmid DNAs were isolated and sent for sequencing. The obtained nucleotide and deduced amino acid sequences were compared again with the Genbank data base. The presence of a signal peptid, indicating the function as a extracellular protein, was also checked using the SignalP server ([www.cbs.dtu.dk](http://www.cbs.dtu.dk)).

	NKA8	NKA48	NKA49
amino acids	257	103	37
signal peptid probability	low (0.352)	no	no
putative motifs		“Greek key”	
significant alignments with known proteins	(1) DUF343 (2) COG2835	(1) $\beta/\gamma$ crystallin (2) spherulin 2b	

**Table 20** Principal characteristics of NKA8, NKA48 and NKA49 after 5'-RACE

As shown in Table 20, one putative motif was found. The so called beta/gamma “Greek key” motifs were first found in crystallins, the dominant structural components of the eye lens. Among the different type of crystallins, the  $\beta/\gamma$  crystallins form a family of related proteins.  $\beta/\gamma$  crystallins are composed of two similar domains, which, in turn, contain two similar motifs. Each motif is folded in the  $\beta/\gamma$  “Greek key” motif, including 4 antiparallel  $\beta$ -strands. Alignment of NKA 48 with the consensus sequence of  $\beta/\gamma$  crystallins was shown in Figure 31. Apart from  $\beta/\gamma$  crystallins, this family also includes the following proteins:

- (1) Protein S, a calcium-binding protein and the major part of the spore coat of *Myxococcus xanthus*.
- (2) Spherulin 2b (previously termed as spherulin 3) from *Physarum polycephalum*, a development specific protein.
- (3) Epidermis differentiation-specific protein (EDSP) of *Cynops pyrrhogaster*.
- (4) Mamalian absent in melano 1 protein (AIM1).

```

consensus 1 KIRLYERENFQGRSYELS---DDCPSLQ-D-RGSRCN-ISSVKVESGH-WVLYERPNIYRG 53
consensus 54 RQYLLEPGEYPDYQDWGG--LNDRISSIRRI 82

alignment:

CD-Length = 82 residues, 95.1% aligned
Score = 54.0 bits (130), Expect = 4e-09

NKA48: 23 VFLYKDANFSGNSWKVTGNVDFRVSGLNDVSSVKVGPNTKAFIFKDDRFNGDFIRLE 82
Sbjct: 2  IRLYERENFQGRSYELSDDCPSLQD-RGSRCNISVKVESGH-WVLYERPNIYRGRQYLLE 59

NKA48: 83 QNTQVTDLTTRNLNDAISSI 102
Sbjct: 60 PGEYPDYQDWGGLNDRISSI 79

```

**Figure 31** Alignment of NKA8 with the consensus sequence of  $\beta/\gamma$  crystallins

Further, the results also showed that NKA 8 belongs to the family of short proteins with unknown function (Figure 32). The bacterial members are about 60-70 amino acids. The eukaryotic examples are about 120 amino acids in length. The C-terminus contains the strongest conservation. 102 proteins containing the consensus sequence of DUF343 are found in different cellular organisms, for example *Pseudomonas aeruginosa*, *Homo sapiens*, *Sulfolobus tokodaii*, *Arabidopsis thaliana* and *Drosophila melanogaster*.

```
consensus  1  DLRLLEILACPVCCKGPLVLVVRKY---DVEEGELICPECGRAYPIRDGIPVMLPDEARD 56

alignment:

          CD-Length = 56 residues,  92.9% aligned
          Score = 41.0 bits (96), Expect = 7e-05

NKA8  :   39   LDRLVCPLDKAALRAHRDDSGKLI-ELVNDRIGVAYPIIRGVPHLTPADARA   89
Sbjct:    5    LEILACPVCCKGPLVLVVRKYDVEEGELICPECGRAYPIRDGIPVMLPDEARD   56
```

**Figure 32** Alignment of NKA8 with the consensus sequence of DUF343

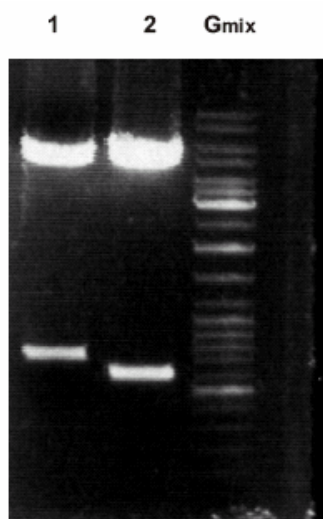
The missing 5'-region of the NKA49 transcript could not be obtained from the 5'-RACE. The experiment was repeated twice with the same results, suggested that the cDNA transcript was probably truncated.



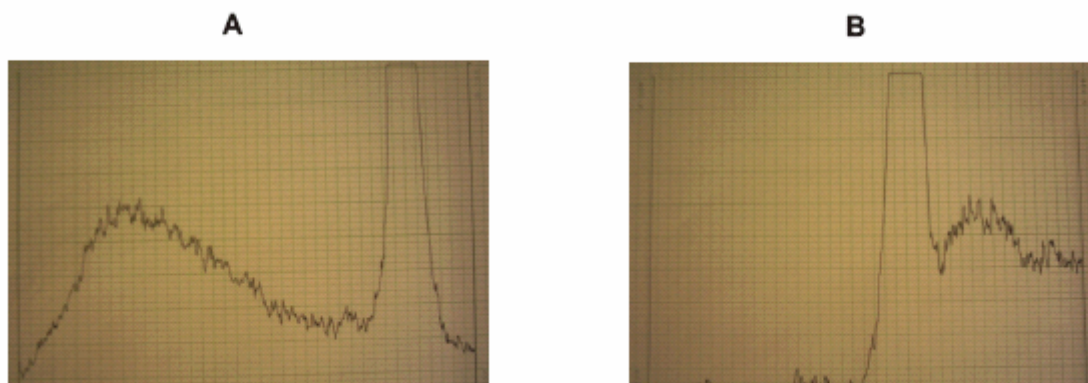
## 2 Knock-down assays of subtracted cDNAs

### 2.1 Verification of EYFP expression in the plasmodium

To elucidate the possible functions of the subtracted cDNAs, methods to down-regulate these transcripts were searched. The first method of choice was an antisense construct including the enhanced yellow fluorescent protein (EYFP) as a reporter gene. First, it has to be clarified whether it is possible to express the cited protein in *Physarum*. For this purpose, vectors contained sense and antisense sequence of EYFP were subcloned and isolated. DNA insertions were verified prior to microinjection into macroplasmodia (Figure 33). Expression of the protein was then detected by fluorimetric measurements. For each time course, two different kinds of negative control were set up: (1) macroplasmodia without microinjection and (2) macroplasmodia injected with a non-fluorescent plasmid, the pBluescript®II SK-vector. Since the excitation maximum wavelength ( $\text{Ex} = 513 \text{ nm}$ ) is very close to that of the emission ( $\text{Em} = 527 \text{ nm}$ ), the protein was excited at an earlier wavelength ( $\text{Ex} = 488 \text{ nm}$ ), to improve the quantification. Fluorimetric measurements of all samples showed the the same pattern (Figure 34).

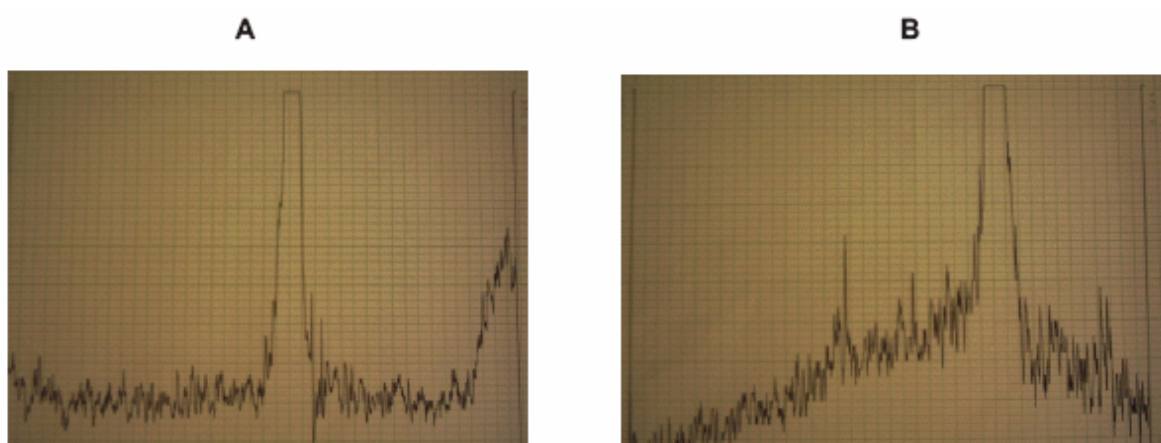


**Figure 33** NcoI/NotI digestion of pJH40-YFP (lane 1) and pJH40-ASEYFP (lane 2),  
G<sub>mix</sub> = GeneRuler™ Ladder mix



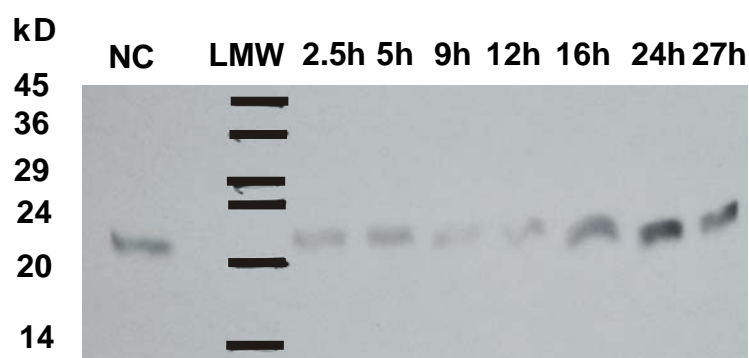
**Figure 34** Fluorimetric measurements of macroplasmidium 12h after injected with EYFP-plasmid. A: Excitation curve by  $E_m = 527$  nm. B: Emission curve by  $E_x = 488$  nm

Both negative controls also showed the same profile, indicating that the fluorescent absorptions were unspecific. To rule out that the lysis buffer contained components, which may cause fluorescence, measurements with exactly the same parameters were performed with the buffer alone. The results owned a different profile (Figure 35), suggested that the fluorescence was probably due to the components included in the cells themselves. Fluorimetric measurements were carried out three times in total with the similar results.



**Figure 35** Fluorimetric measurements of total cell lysis buffer. A: Emission curve by  $E_x = 488$  nm. B: Excitation curve by  $E_m = 527$  nm.

It has been reported, that in some case, GFP expression constructs could not be detected in a fluorescence spectrophotometer [Clontech, Living Colors® User Manual 2001]. There can be several reasons for failure, including expression below the limit of detection or failure of the protein to form the chromophore. Using specific antibody may provide better alternative for detection of the proteins. Hence, Western Blots with specific antibody against enhanced GFP variants including EYFP were next performed to monitor the expression. First Western blot was carried out using 100 µg proteins from the total cell lysis. Since no bands were observed the experiment was repeated once with 500 µg proteins. As shown in Figure 36, a distinct band, lower than the expected size (about 30 kD), could be observed overall, even in the negative control (without microinjection), supporting the assumption that the fluorescence observed may cause by components including in the cell, probably a protein with similar structure to EYFP.



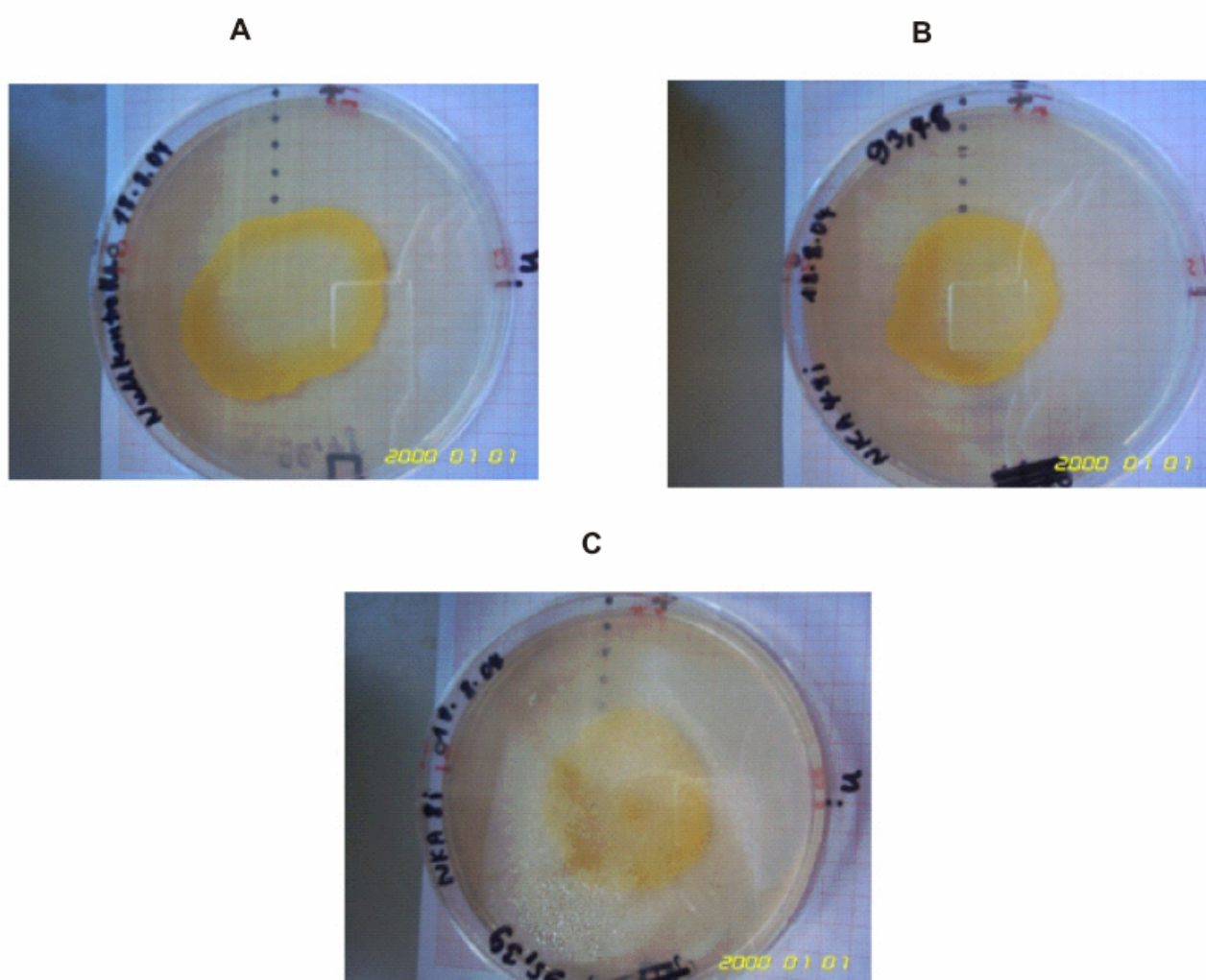
**Figure 36** Western Blot using specific antibody against GFP and variants

## 2.2 Knock-down assays using ds RNA

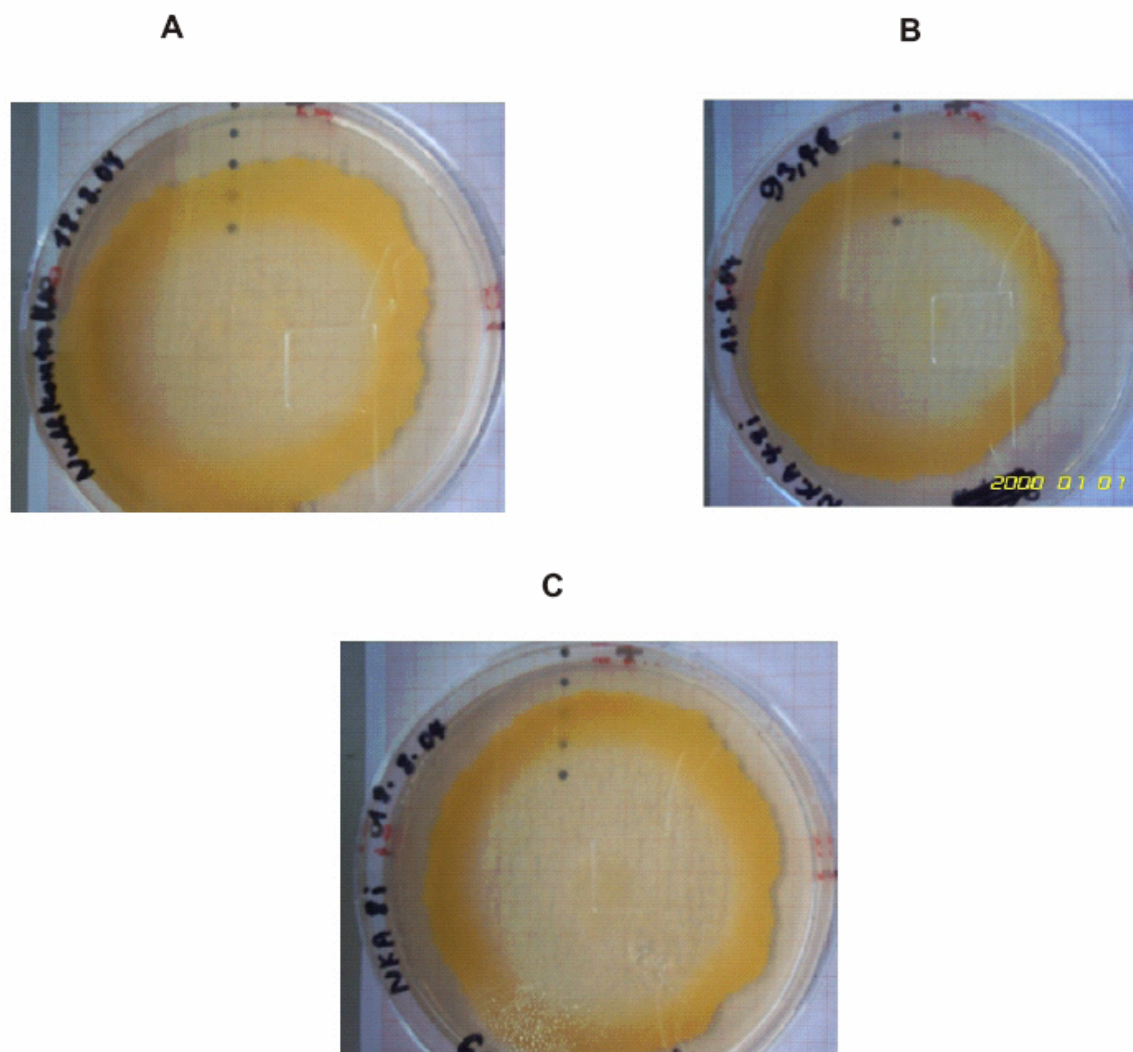
### 2.2.1 Verification of RNAi effect by monitoring phenotypically change

Since the fluorescent protein EYGF could be detected neither by fluorometric measurement nor by Western Blotting, a new possibility to down-regulate the differentially expressed transcripts (NKA8, NKA48 and NKA49) was search and found in dsRNA. The efficiency of dsRNA is usually depends on the length of this

molecule. For successful gene silencing, dsRNA should be at least 500 bp. Since NKA49 was too short to serve as template, only NKA8 and NKA48 were *in vitro* transcribed into RNA. Knock-down analysis was carried out by monitoring development of the cells after microinjection. Microplasmodia were allowed to grow at 24°C in the dark. 24h, 48h, 96h and 120h after microinjection, half of the plate was harvested and frozen in liquid nitrogen for later analysis. After 120h the plates were allowed to grow until any morphologically change was visible. The results are shown below.

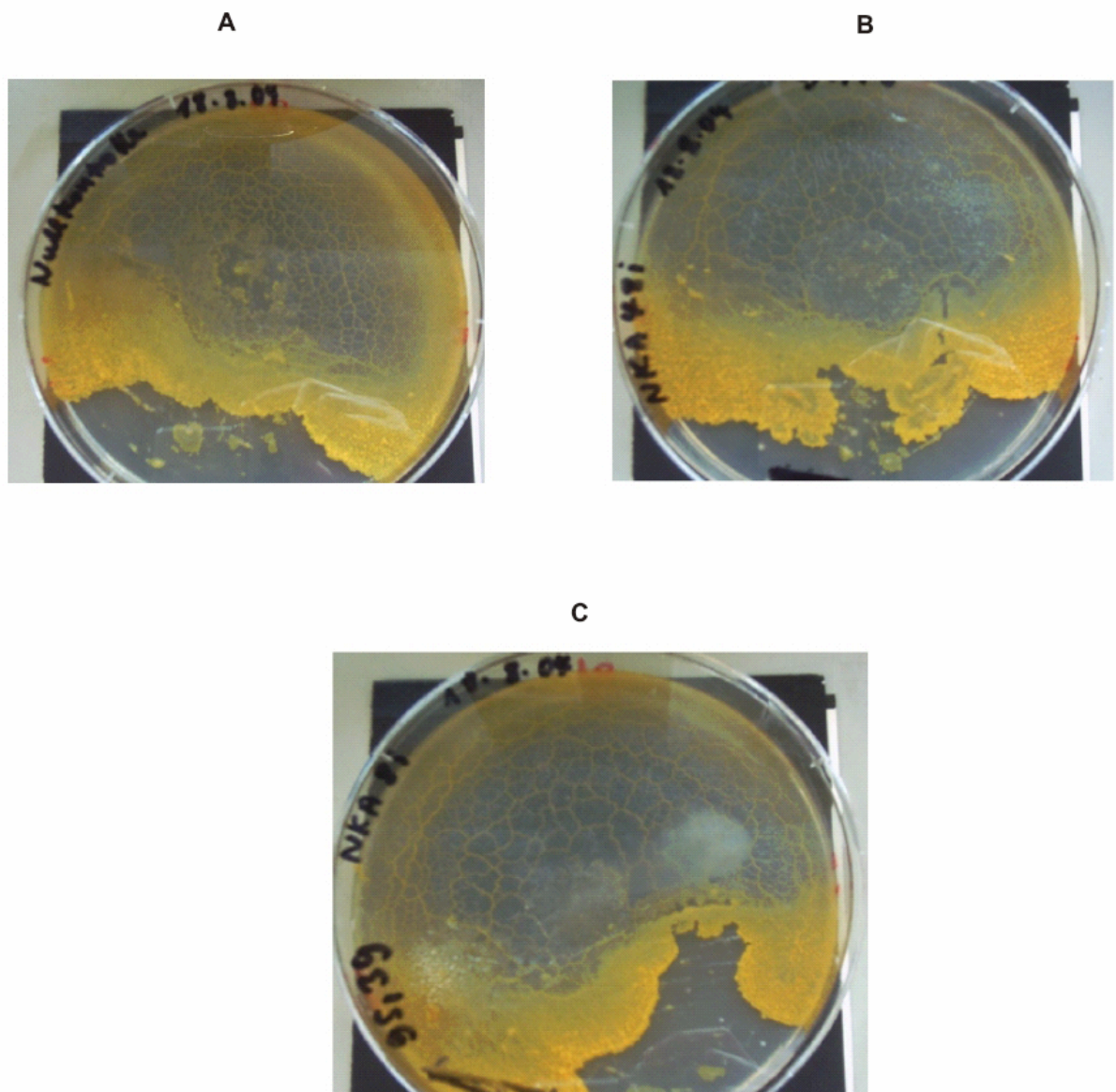


**Figure 37** Microplasmodia before microinjection. A: Microplasmodia prepared for negative control (without microinjection) B: Microplasmodia prepared for injection with 48i-dsRNA. C: Microplasmodia prepared for microinjection with 8i-dsRNA.

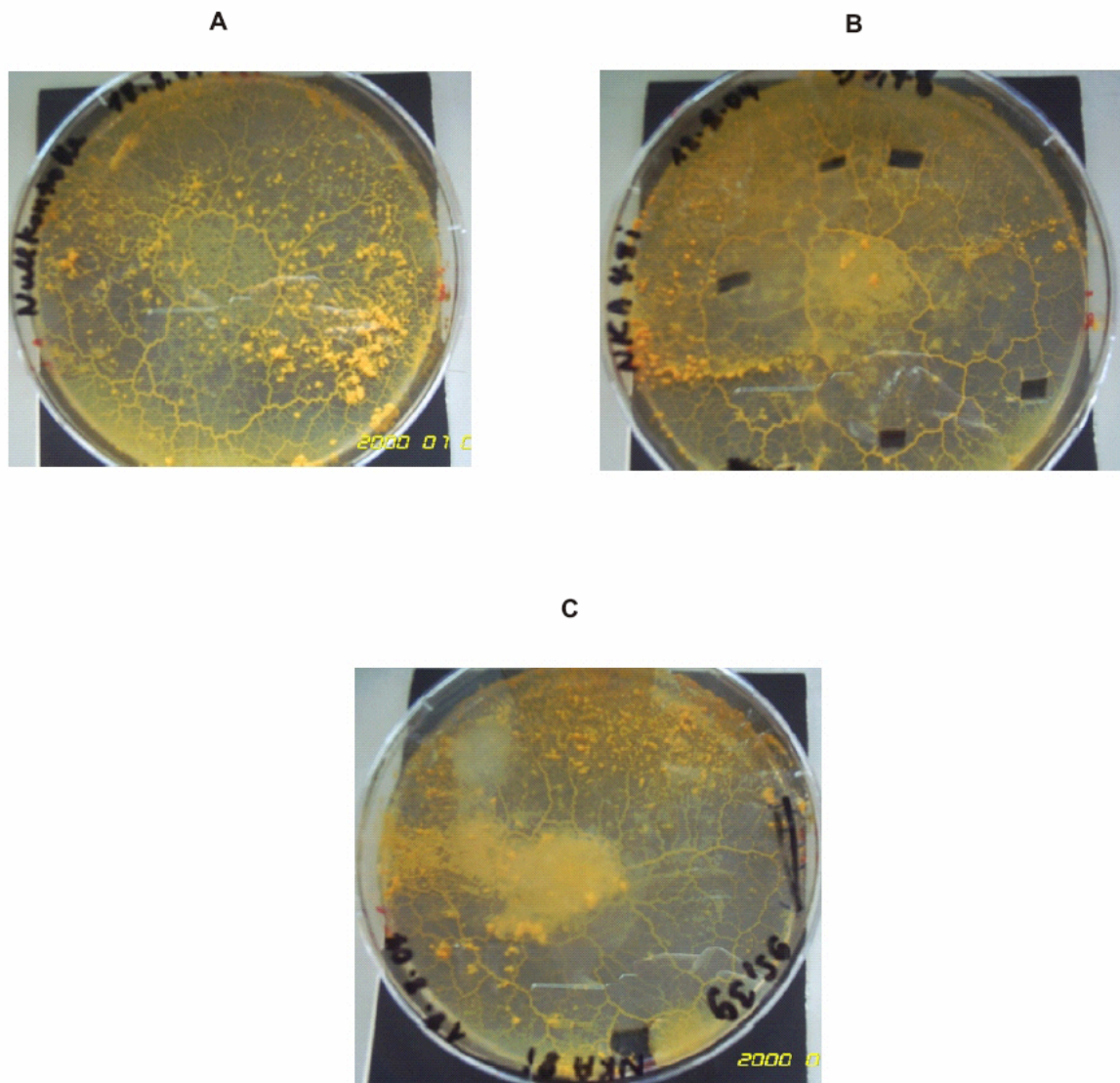


**Figure 38** Microplasmodia 24h after microinjection. A: Negative control. B: Microplasmodia injected with 48i-dsRNA. C: Microplasmodia injected with 8i-dsRNA.

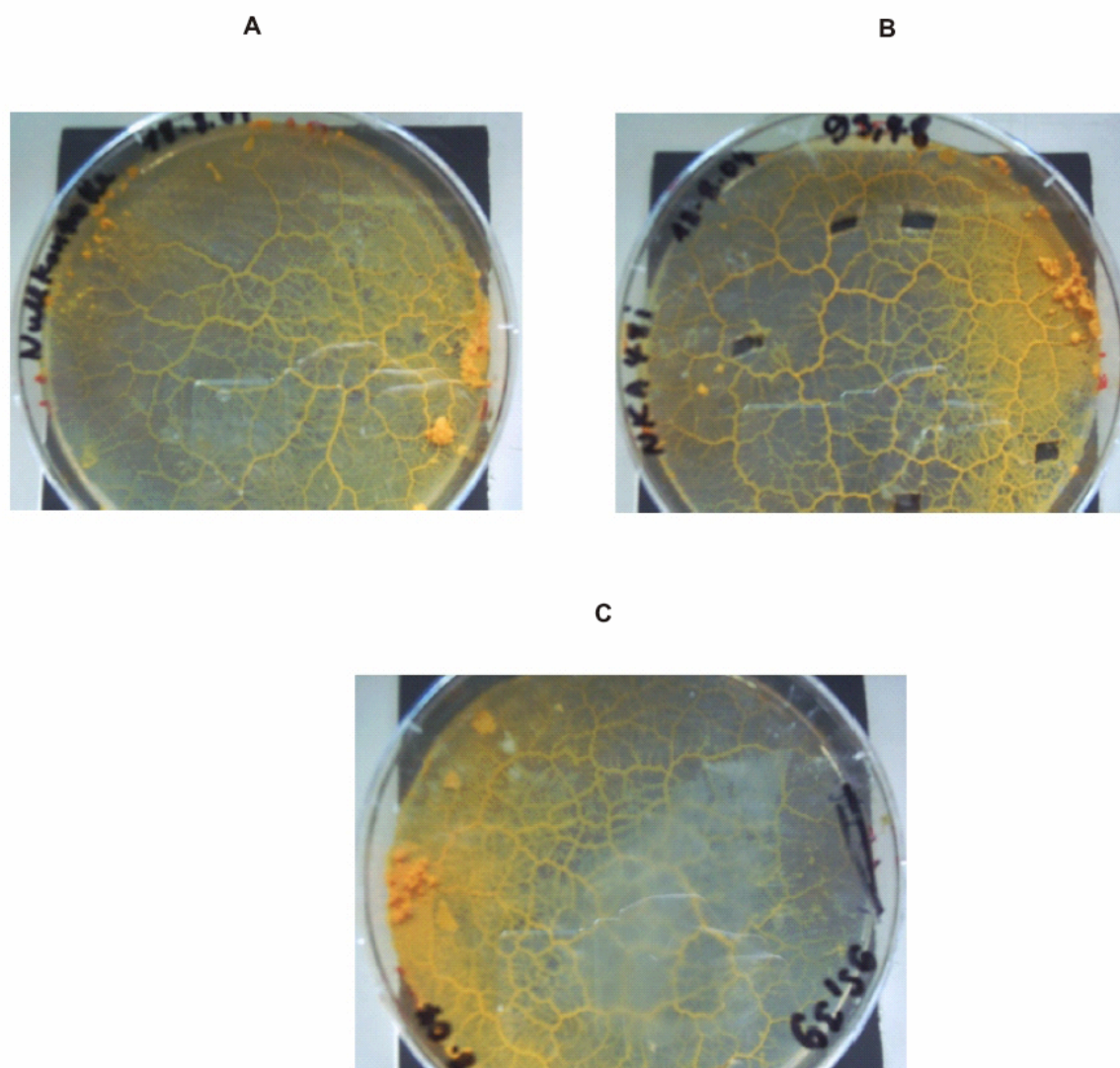




**Figure 39** Microplasmodia 48h after microinjection. A: Negative control. B: Microplasmodia injected with 48i-dsRNA. C: Microplasmodia injected with 8i-dsRNA.

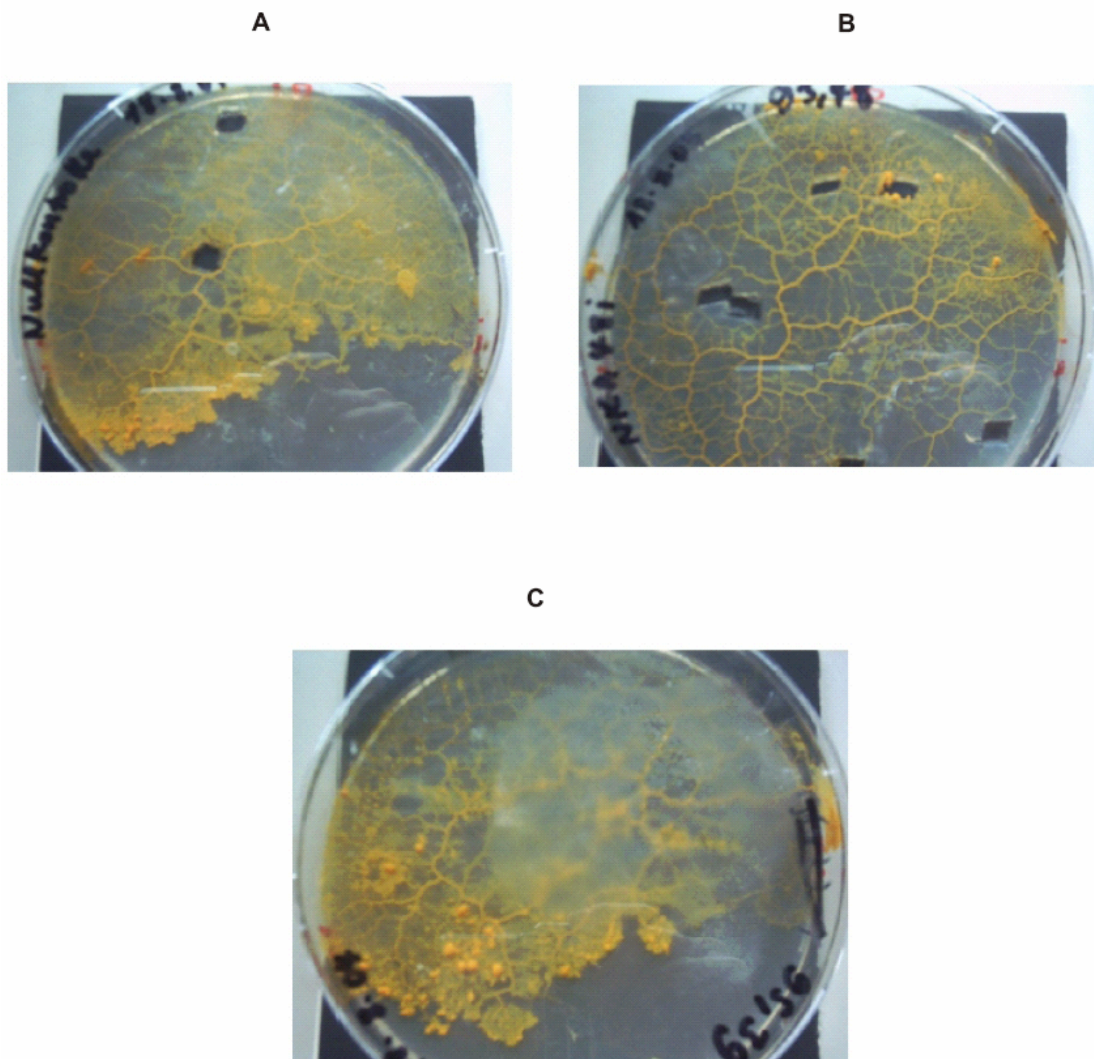


**Figure 40** Microplasmodia 96h after microinjection. A: Negative control. B: Microplasmodia injected with 48i-dsRNA. C: Microplasmodia injected with 8i-dsRNA.

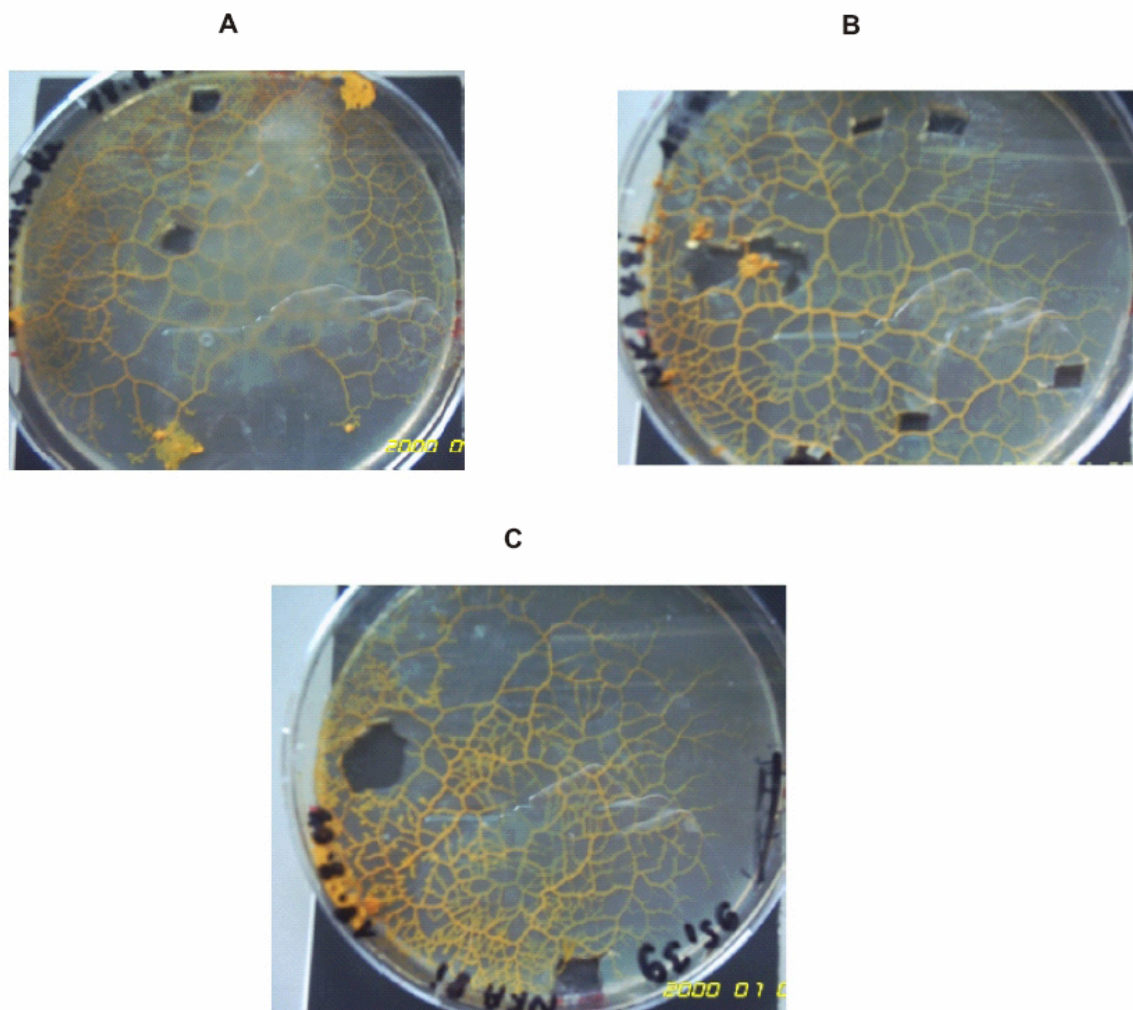


**Figure 41** Microplasmodia 4 days after microinjection. A: Negative control. B: Microplasmodia injected with 48i-dsRNA. C: Microplasmodia injected with 8i-dsRNA.

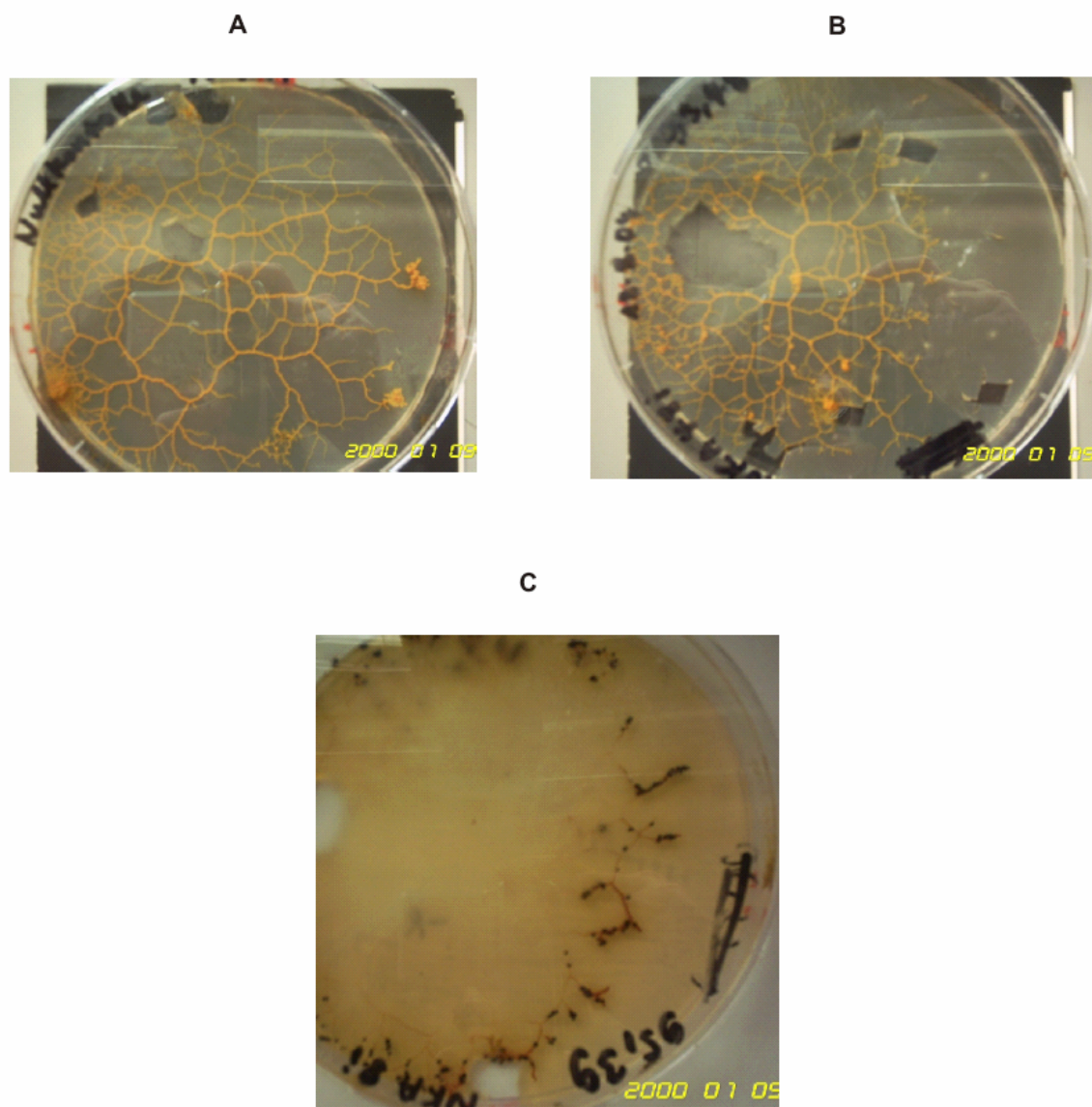




**Figure 42** Microplasmidia 5 days after microinjection. A: Negative control. B: Microplasmidia injected with dsRNA derived from NKA48 cDNA transcript. C: Microplasmidia injected with dsRNA derived from NKA8 cDNA transcript.



**Figure 43** Microplasmodia 7 days after microinjection. A: Negative control. B: Microplasmodia injected with 48i-dsRNA. C: Microplasmodia injected with 8i-dsRNA.

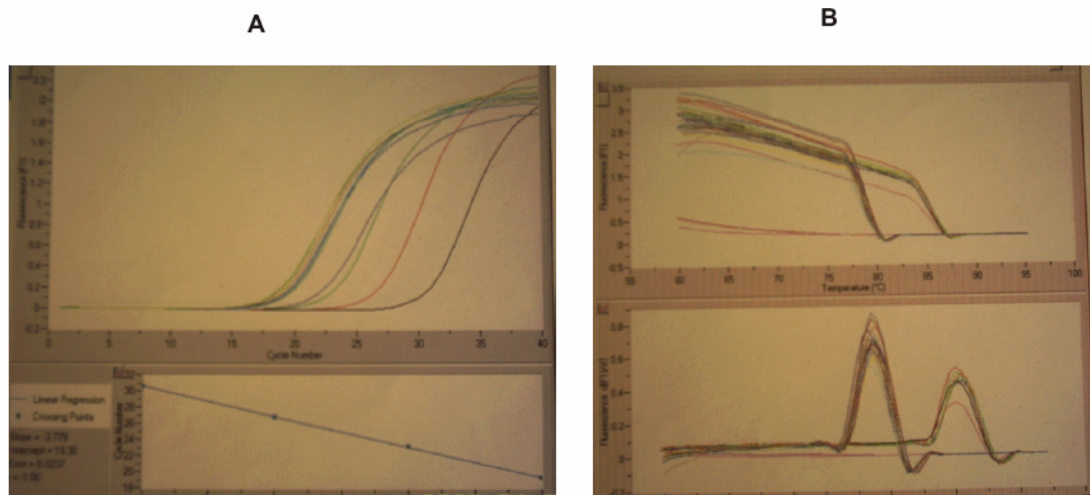


**Figure 44** Microplasmodia 10 days after microinjection. A: Negative control. B: Microplasmodia injected with 48i-dsRNA. C: Microplasmodia injected with 8i-dsRNA.

The results showed that macroplasmodia injected with 48i-dsRNA seem to lose the ability to regenerate after the last harvest, since they showed the first signs of old age (the veins became salient), while other cells partially remained in the growth phase (Figure 42). At the 7<sup>th</sup> day, all of the plasmodia reached the starvation stage. Thus, macroplasmodia injected with 48i-dsRNA reached the starvation stage two days earlier than the others. Although the aging process of macroplasmodia injected with 48i-dsRNA was obviously faster than those of other cells, they were unable to sporulate. The sporulation was exclusively observed in the microplasmodia injected with 8i-dsRNA after 10 days. After 14 days, other cells were dead, while “8i-dsRNA”-plasmodia demonstrated the same extent of starvation. The experiments were finished and repeated twice, resulting in the similar pattern. Since microinjections were not carried out under pyrogen free condition, small contaminations caused by spores in the air were nearly unavoidable. However, effects of these contaminations on the cells resulting in the phenomenon observed can be ruled out, since contaminations were found in all samples and were removed before the whole plate was reached. Thus, the contaminations were only punctually.

### 2.2.2 Quantification of RNAi effect by Real-time PCR

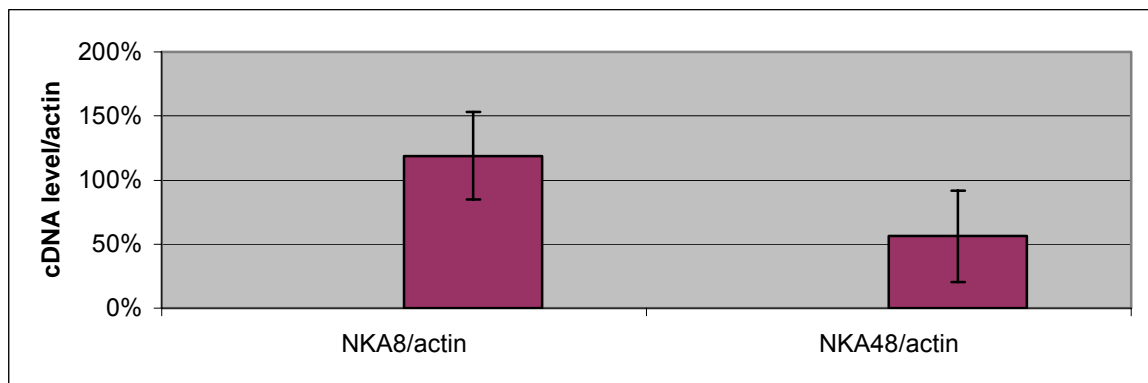
Knock-down analysis was also performed by Real-time PCRs in the LightCycler System. Quantification of NKA8 and NKA 48 after microinjections was carried out with cDNAs isolated from 4 days old frozen microplasmodia. Figure 45 demonstrates one of the typical profiles obtained from the quantification of target cDNAs after microinjections. The melting curves demonstrated two different specific products. No primer-dimers were formed, since no peak was observed at lower temperatures.



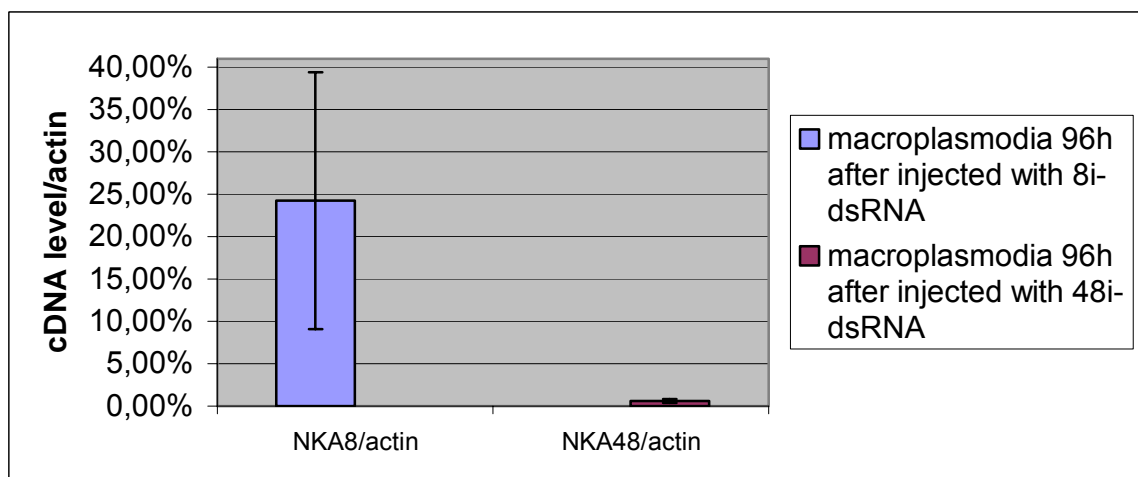
**Figure 45** Real-time PCRs after microinjections. A: Amplification plots. B: Melting curves.

Amplification plots indicated that the ratio of NKA48 to the housekeeping gene, actin, was significantly reduced, to less than 1%, in microplasmodia injected with 48i-dsRNA (Figure 46). In contrary down regulation of NKA8 was much less effective (Figure 46, 47). Knock-down experiments with polymalatae, which show about 1:1 ratio to actin before injection, resulted in down regulation of the target to less than 10% [Haindl, diploma thesis, 2004]. Thus, it is unlikely that the higher ratio of NKA8 to actin at the beginning (comparing with that of NKA48) may be the reason for the less efficiency. The mechanism of gene-silencing by dsRNA has not been yet completely understood. As shown in Figure 47 the standard deviation is much higher than received before the injection, suggested that down regulation of the transcript was differently efficient in the three samples. Hence, the efficiency of gene silencing by dsRNA may depend on certain not yet known factors.





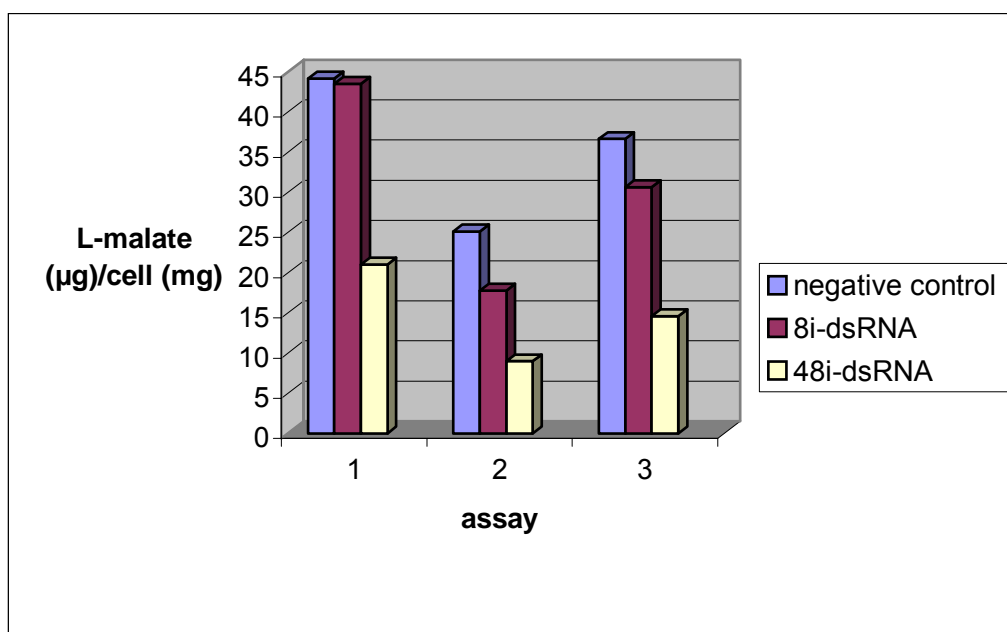
**Figure 46** cDNA level of NKA 8 and NKA 48 relative to actin in macroplasmodia without microinjection.



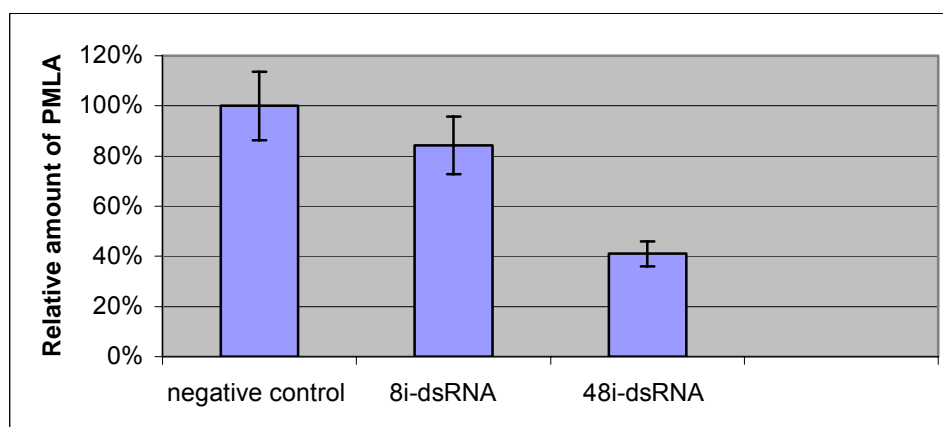
**Figure 47** cDNA level of NKA 8 and NKA 48 relative to actin in macroplasmodia, 96h after microinjection.

### 2.2.3 Knock-down analysis by quantification of PMLA level

To clarify whether NKA8 and NKA48 also involved in the production of PMLA, the amount of the polymer was quantified after microinjections. It has been observed that excess of PMLA is transported into the liquid culture after 4-5 days. Hence, to ensure measurable amount of the polymer in the cells, fluorimetric measurements were carried out with the 2 days old macropasmodia. The results showed that the production of polymalate was significantly reduced (up to 40%), when the cells were injected with dsRNA derived from the NKA48 transcript (48i-dsRNA, Figure 48, 49). In contrary, only little change was observed in the presence of dsRNA transcribed from the NKA8 template (8i-dsRNA, Figure 48, 49). Thus, a coherence between spherulation and PMLA is most likely. Since polymalase activity in cytoplasm extracts is only marginally due to unfavourable pH, it has been proposed that the enzyme is released together with the polymer into the culture medium in response to unfavourable conditions such as starvation. Hence, PMLA may not only function as a storage and transport system for nuclear proteins but may also involve in regulation of growth and sperulation.



**Figure 48** Quantification of PMLA after microinjection.



**Figure 49** PMLA productions in injected macroplasmodia, comparing with the negative control.

### 3 Quantification of polymalatase at cDNA level

The level of polymalatase cDNA transcript during the life cycle was monitored by using Real-time PCR in. Since it has not been ruled out that cDNA level of actin may vary from cell type to the other, absolute quantification seems to be more reliable. A PCR fragment containing the target sequence was used as a standard. The initial amount of the target was calculated by interpolation into the standard curve. For each cell type, three different cDNA preparations were used for the measurement. Table 21 shows the determined concentrations of target cDNAs at different stages of the life cycle.

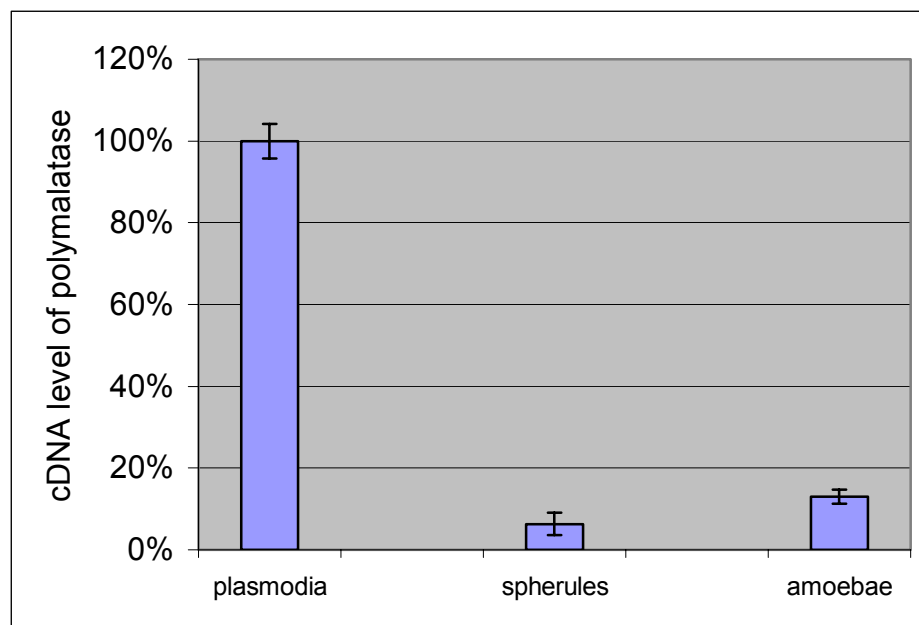
samples	cDNA (g/μl)	relative to plasmodium	in average	A)
plasmodium 1	8.67E-15			
plasmodium 2	9.90E-15			
plasmodium 3	8.17E-15			
spherules 1	7.28E-17	8.39%	6.33%	
spherules 2	7.34E-17	7.41%		



spherules 3	2.61E-17	3.20%	
amoebae 1	1.12E-16	12.90%	13.03%
amoebae 2	1.47E-16	14.80%	
amoebae 3	9.30E-17	11.40%	

**Table 21** Quantification of polymalatase cDNA in plasmodium, spherules and amoebae

A) For standard deviations, see Figure 50



**Figure 50** cDNA level of polymalatase among different cell types of the life cycle.

As shown in Table 21 and Figure 50, the level of target cDNA significantly decreased from the plasmodial to the amoebal stage. In the case of spherules only trace amount of polymalatase was detected. Standard deviations were small in all cases, indicated that the difference in cDNA level is significant. Together with the evidence that the encoded protein could be detected neither in amoebae nor in spherules suggested that the regulation of polymalatase is cell-type specific and probably occurs at transcriptional level.

## V Discussion and outlook

### 1 Suppression subtractive hybridization

The evidence that  $\beta$ -poly(L-malate) is found only in the growing plasmodium but not in any other stages of the life cycle, may be result of difference in gene expression. To gain deeper insight into the regulation of polymalate production, differentially expressed genes were identified using the so-called suppression subtractive hybridization. The method promises a powerful enrichment for differentially expressed transcripts obtained in rather small cDNA libraries. To estimate the efficiency of the subtractive hybridization, the abundance of actin transcripts was checked before and after subtractive procedure. PCR analysis showed the amplified product in the subtracted sample 5 cycles later, suggested that the subtractive procedure worked well. The evidence that no actin transcript was obtained from the subsequent cloning procedure also confirmed the success of the subtraction. The suppression subtractive hybridization was combined with the so-called CapFinder cDNA synthesis, which provides full-length cDNAs from the little amount of the starting material. However, the results showed that the 5'-end of the cDNAs including possible start codons was missed in most case, indicating that the tagging of this region was not successful. When the reverse transcriptase reaches the 5' end of the mRNA, the terminal transferase activity of the enzyme usually adds 2-4 additional nucleotides, primarily deoxycytidine, to the 3'-end of the cDNA. Since the first-strand synthesis was oligo(dT) primed, it is likely that the reverse transcriptase paused before the end of the templates, resulting in addition of deoxycytidine with less efficiency. Thus, the base-pairing with the CapFinder oligonucleotide was prevented and poly C, which probably included in some cDNA sequences were bound instead.

PCR analysis indicated that three of the subtracted cDNAs were plasmodial specificity, since they were not detected in amoebae. To obtain the missing 5'-region of these differentially expressed cDNAs, 5'-RACE was performed using a gene-specific primer and terminal deoxynucleotidyl transferase (TdT). TdT usually adds 6-8 residues to the 3'-end of the cDNA. Thus, the subsequent annealing of oligo(dG) primer (CapFinder) should be more specific, since the presence of up to 8 G residues

in a cDNA sequence is unlikely. 5'-RACE successfully extended NKA8 and NKA48 but failed to detect the 5'-region of NKA 49. Since the resulting DNA sequence was similar to that obtained from the CapFinder cDNA synthesis. 5'-RACE was then repeated twice without success, suggested that the cDNA was truncated. There can be several reasons for the failure: (1) mRNA transcript may be degraded. or (2) probably includes a persistent secondary structure, leading to prematurely terminated cDNA synthesis. (3) The mRNA is rudimentary and does not encode a functional protein. The latter seems to be most likely, since comparison with the GenBank database showed neither putative motifs nor significant alignments with known proteins. In contrary, NKA48 included the highly conservative “greek key” motif, which was first found in  $\beta$  and  $\gamma$  crystallins. Apart from the different types of  $\beta$  and  $\gamma$  crystallins, five other proteins, including Spherulin 2b, also belong to this family. Furthermore, comparison of the nucleic and deduced amino acid sequence with the GenBank demonstrated high homology of NKA48 to spherulin 2b. However, NKA48 is about 20 bp shorter than spherulin 2b. Since the non-coding region of NKA48 seems to be very short, cDNA synthesis was probably not complete. Hence it is most likely that NKA48 and spherulin 2b are one and the same gene. Significant alignment was also found in the case of NKA8. The deduce amino acid sequence of this transcript, includes consensus sequence of DUF343, the family of short proteins with unknown function. In total, 102 proteins containing the consensus sequence of DUF343 were found in different organisms. NKA8 was the first one found in *Physarum polycephalum*.

## 2 Knock-down assays of the subtracted cDNAs

### 2.1 Verification of EYFP expression in the plasmodium

Several substances have been incorporated into living cells. Three methods have been established to introduce substances into the cytoplasm of *Physarum polycephalum*:

- (1) Cell membrane perforation by treatment with detergents, e.g. Triton (Helenius and Simons, 1975). Membrane perforation induces both outflow of soluble cytoplasmic components and inflow of applied substances. Insoluble cytoplasmic components such as the actinmyosin system are retained in the cell.
- (2) Electroporation. This method have been found to be successful for introducing functional DNA into amoebae.
- (3) Microinjection. Microinjection by capillaries is in most case preferable, since this method does not destroy the integrity of the target cell. Nearly all substances have been successfully microinjected into macro and microplasmodia.

Reporter genes, which code for enzymes with readily-detectable activities, are routinely used to monitor gene expression, and related cellular events. Hitherto analyses of gene regulation in *Physarum polycephalum* were carried out in amoebae, since clones of stable transformants could be easily obtained from this cell typ. In most case the reporter constructs were successfully transformed to the cells by electroporation [Burland and Bailey, Methods in Molecular Biology, Vol. 47]. However, little is known about transformation of plasmid DNA to plasmodia. To verify the utility of microinjection for introducing plasmid-DNA into plasmodia a new reporter construct was applied. The expression of the encoded fluorescent protein was monitored by both fluorimetric measurement and Western Blotting. Results from both

procedures indicated that the construct failed to express a functional fluorescent protein, since no specific signal was detected. Two possible reasons for failure may be (1) plasmid DNA was not successfully introduced to the cells or (2) the included promoter did not work. However, the latter seems to be unlikely, since the plasmid carried one of the strongest promoters tested as far, namely the promoter for the *ardC* actin gene. This gene is the most highly expressed of the *Physarum* actin multigene family and is expressed in both amoebae and plasmodia. It has been reported that in some cases, proteins such as fluorescently labeled actin or albumin were rapidly sequestered into numerous vacuoles after injection, and were thus prevented from reaching the peripheral ectoplasm [Kukules et al., 1984]. Hence, it is likely that microinjection failed to introduce the plasmid DNA, resulting in no expression of the fluorescent protein EYFP.

## 2.2 Knock-down assay using dsRNA

The encystment of plasmodia, also called spherulation, involves the synthesis of many specific mRNAs and proteins. The major changes in protein synthesis usually take place 24h after starvation. Four highly abundant spherulation-specific mRNAs were detected at this period. These mRNAs were not present in encysting amoebae and sporulating plasmodia. The most abundant spherulation-specific mRNAs among the four cited transcripts is spherulin 2b. The encoded protein is composed of 103 amino acids and does not contain an *N*-glycosylation signal nor a transit peptid, suggesting that it belongs to structural proteins. As already mentioned, the differentially expressed transcript NKA48 is highly homologous to spherulin 2b, when not identical. Macroplasmodia injected with dsRNA derived from NKA48 (48i-dsRNA) showed no striking morphological difference compared with other cells, except that the aging process of these cells became a little more rapid. In contrast, the effect of 48i-dsRNA was remarkable with regard to the production of poly(L-malate). It was found that the production of this polymer was significantly reduced to less than 50%, in the presence of 48i-dsRNA. Since suppression of NKA48 cDNA was confirmed by Realtime PCR, the decrease of PMLA production was most likely due to the gene silencing of the spherulin 2b homologous NKA48. Together with the assumption that the degrading hydrolase is transported into the culture medium with

response to unfavourable condition [Korher et al., 1995], suggested that poly(L-malate) may also play an important role in induction of sporulation.

Sporulation usually occurs when plasmodia are starved in the light. The morphological differentiation of plasmodial veins into fruiting bodies starts about 10 hr after the beginning of illumination. It has been found that sporulation is controlled by a signal-transduction pathway including specific receptors for blue and far-red light, heat shock and the nutritional status of the cell [Starostzik and Marwan, 1995]. In contrary, less is known about sporulation in the dark. Merely Wormington and Weaver (1976) have claimed that one of the wild-type plasmodial pigments is probably involved in the photoinduction in the unilluminated plasmodia, since injection of the extract containing this molecule resulted in sporulation of starved, unilluminated cells. However, these studies have not been confirmed. Knock-down assays showed that only plasmodia injected with dsRNA derived from the NKA8 transcript were able to sporulate in the dark. Since comparison of NKA8 to the Genbank database indicated that the encoded protein belongs to the family of short proteins with unknown function, one can only speculate about possible role of NKA8 in Sporulation. Since no remarkable change in the production of PMLA was observed in the presence of 8i-dsRNA, a connection between the polymer and the transcript seems unlikely. NKA8 may not be directly involved in the sporulating process but probably interact with other components resulting in the observed sporulation. Further analyses, such as monitoring of changes in protein distribution, are required to clarify the role of this protein.

### **3 Quantification of polymalate at cDNA level**

Many regulatory events occurring in processes such as cell differentiation, embryo development, or malignant transformation depend on activation and/or suppression of specific genes. Generegulation can occur at either transcriptional or translational level. Transcription of a gene can be affected by sequence-specific binding proteins. Most of these transcription factors usually include conserved consensus sequences,

such as TATAA box and a GC-rich region. Analysis of promotor region of polymalatase showed multiple sequences homologous to known transcription binding sites [Haindl, diploma thesis 2004], including Sp1 (transcription factor for ornithine decarboxylase in mammalian HeLa-cells, Li et al., 1994), GHF-7 (transcription factor for rat growth hormone, Schaufele et al., 1990) and *dl*, a transcription factor for dorsal protein in *Drosophila* [Ip et al., 1991]. The presence of multiple binding sites of transcription factors together with the absence of the encoded protein in amoebae and spherules suggested that there might be a regulation of the gene at the transcriptional level. Real-time PCR analysis demonstrated that cDNA level of Polymalatase was significantly reduced in amoebae and plasmodia, thus supporting this assumption. Since these both cell types do not produce polymalate, down regulation of polymalatase seems to be a logically consequence. Since amoebae and spherules can develop into plasmodia, a certain level of polymalatase may be required for this transformation and thus kept constant, probably about 5-10% of that usually produced in plasmodia.

## 4 Outlook

Knock-down assays using dsRNA derived from two differentially expressed transcripts resulted either in remarkably decrease of PMLA production or in ability to sporulate. However, since part of the cells were harvested every 24h, it cannot be ruled out that these procedures may also affect on the growth of the cells. Hence, to confirm that the effects observed are specifically induced by the derived dsRNA, the same experiments should be carried out without harvesting the cells. Further, knock-down assays with non-specific dsRNAs may clarify whether NKA 8, one of the 102 short proteins without known function, plays an important role in sporulation in the dark or is it only an appendage of the evolution.



## VI Conclusion

The acellular slime mold *Physarum polycephalum* is characterized by two distinctive growth phases: uninucleate amoebae and multinucleate syncytial plasmodia. These two cell types differ in cellular organization, behaviour and gene expression. In adverse conditions, plasmodia reversibly transformed into spherules, when starved in the light sporangia are formed. Plasmodia distinguishes from other stages of the life cycle by the production of an unusual polyester,  $\beta$ -poly(L-malate) (PMLA). The polymer is concentrated in the nuclei and has been proposed to function as storage molecule and a mobile matrix for nuclear proteins. PMLA may also play a role in growth and synchronization in plasmodia. However, little is known about genes involved in the biosynthetic pathway of this polymer. Merely, the gene encoded for the degrading enzyme (polymalatase) is nearly characterized. The absence of  $\beta$ -poly(L-malate) in other cell types, suggested a difference in gene expression. Hence, the aim of this work was to identify differentially expressed genes, which may play a role in the synthesis and degradation of the polymer. Further, it was to clarify whether the synthesis of polymalatase is regulated at transcriptional or translational level.

Three plasmodial-specific transcripts were obtained after the so-called suppression subtractive hybridization. This method is based on the suppression effect of inverted terminal repeats in a polymerase chain reaction. These inverted repeats namely form stable pan-like loop structures after each denaturation and annealing cycle, preventing the exponential amplification of these molecules. Thus, if inverted repeats are attached to undesirable DNA fragments, in this case, commonly expressed transcripts, undesirable DNAs can be eliminated from the mixture of target sequences. To elucidate possible functions of the differentially expressed cDNAs, knock-down assays were carried out by both dsRNA and antisense construct including a reporter gene. Expression of the encoded reporter protein could not be detected, suggesting that microinjection failed to introduce plasmid DNA to the cells. One possible reason for the failure may be the phenomenon of sequestration, which was observed by injection of labeled actin or albumin [Kukulies et al., 1984]. The other reason may be failure of the promoter to express functional protein. In

contrary, knock-down assays using dsRNA were successful, resulting either in morphological change or decrease of  $\beta$ -poly(L-malate) production. One of the plasmodial-specific transcripts obtained, showed a consensus sequence of a protein family of unknown function. Knock-down assays indicated that only macroplasmodia injected with dsRNA derived from this transcript were able to sporulate in the dark. The second subtracted transcript showed high homology to the spherulation-specific spherulin 2b. Down regulation of this gene significantly reduced the production of PMLA suggested that there may be a connection between the polymer and dormancy. PMLA probably plays an important role in the induction of spherulation. Further analyses are required to confirm the specificity of the effects observed, e.g., knock-down assays with non-specific dsRNAs.

PMLA hydrolase is the only one enzyme from the biosynthetic pathway that is well characterized. Genomic DNA sequence demonstrated multiple sequences binding sites homologous to known transcription factors. Real-time PCR analysis of polymalatase transcript showed that the level of cDNA was significantly reduced in amoebae and spherules. Together with the presence of several transcription binding sites, it is likely that polymalatase is regulated at transcriptional level.

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## cDNA sequences



10



**I** = 3'-T overhang from pGEM<sup>®</sup>T-vector

DSSQINAKFTDGVLDLTIPKPAIKEPEKKKIEVK **Stop** ADVSL  
RHRPAFIS **Met** YN **Stop** LCTVNKIICISKKKKKKKKKKKKKKKK  
KKKKKKKKKKKKKKKKKKKKKKKKKK

[5'3' Frame 2](#)

THRRSTPSSLTV C **Stop** T **Stop** PSLSPPSRSPRRRRSRSSKLT  
CPYVIARHL **Stop** ACITNYVP **Stop** IKLYVFLKKKKKKKKKKKK  
KKKKKKKKKKKKKKKKKKKKKKKK

[5'3' Frame 3](#)

LIADQRQVH **Stop** RCVRPDHP **Stop** ARHQGAREEEDRGQVS  
**Stop** RVLTS SPGIYKHV **Stop** LI **Met** YRK **Stop** NY **Met** YF **Stop** KKKK  
KKKKKKKKKKKKKKKKKKKKKKKKKKKKKK

[3'5' Frame 1](#)

FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFRNTYNFIYG  
T **Stop** LVIHAYKCRA **Met** T **Stop** GHVSLLDLLLLGLLDGGLR  
DGQV **Stop** HTVSELGVDLR **Stop** V

[3'5' Frame 2](#)

FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFLEIHIILFTVH  
N **Stop** LY **Met** LINAGR **Stop** RKDTSAYLTSIFFFSGSL **Met** AGLG  
**Met** VRSNTPSVNLALICDE

[3'5' Frame 3](#)

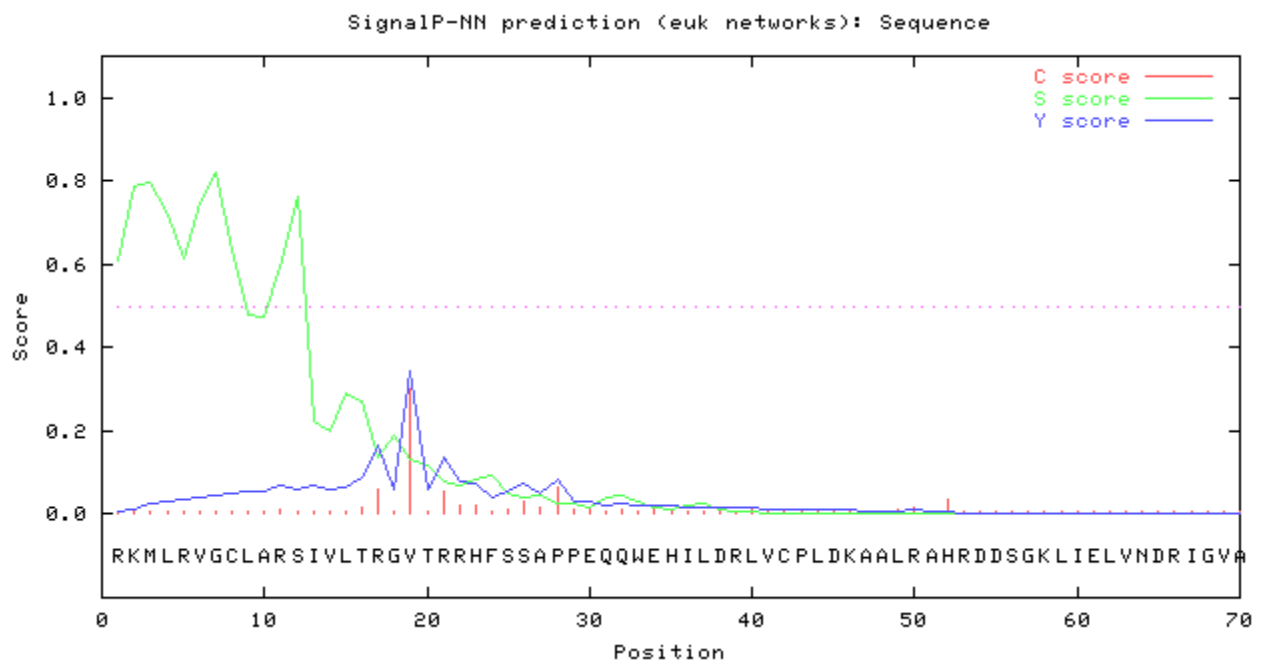
FFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFFF **Stop** KYI **Stop** FY  
LRYIISYTCL **Stop** **Met** PGDDVRTRQLT **Stop** PRSSSSRAP **Stop**  
WRA **Stop** GWSGLTHRQ **Stop** TWR **Stop** SA **Met** S

**NKA 8** (complete cDNA-sequence after 5'-RACE, cap- and adaptor-sequence are not shown)

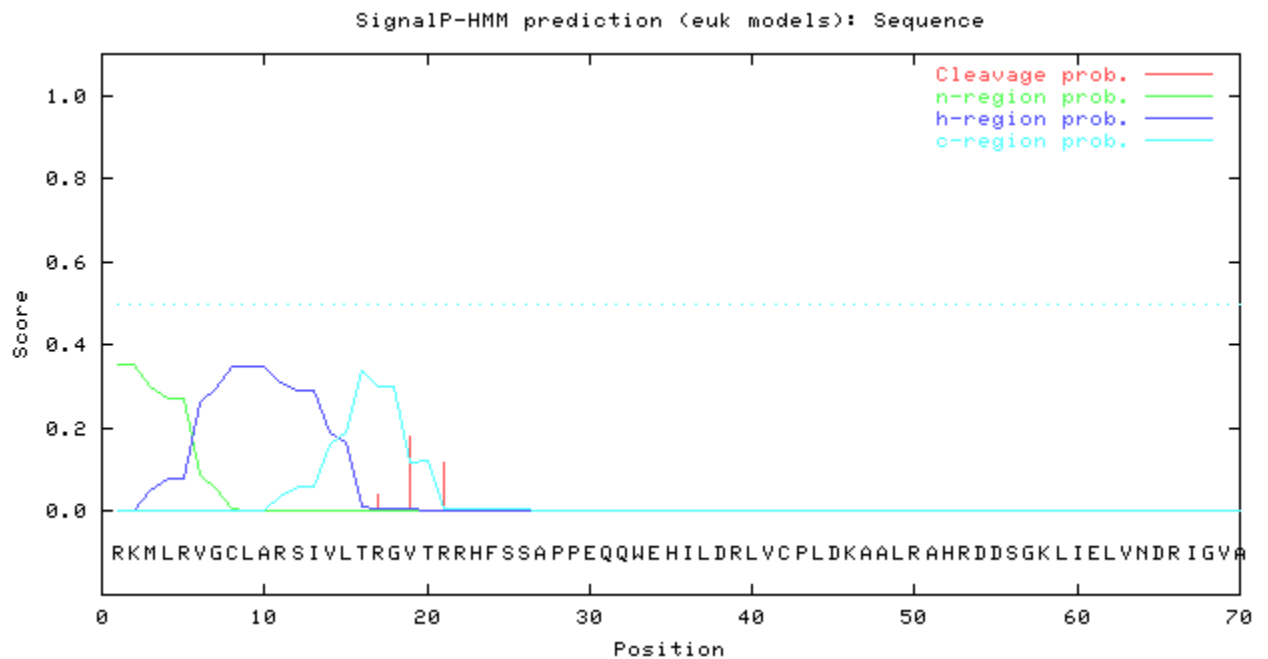
```
CGCAAAATGCTGCGCGTTGGGTGCCTTGCAAGGAGTATTGTTCTCACACGTGGCGTTACT 60
AGAAGGCACTTTAGCTCTGCACCCCCAGAACAGCAATGGGAGCACATATTAGATAGACTT 120
GTTTGCCCACTTGATAAAGCTGCCCTGCGCGCGCACCGTGACGACTCGGGAAACTCATA 180
GAGCTTGTCAACGACCGGATAGGGGTTCATACCCGATAATACGTGGCGTTCCCCATCTT 240
ACACCAGCCGACGCGCGTGCCCTCAACCCGGCAGCCAGCAGAGCATGAAGGCCGCTCAG 300
AAGGGAGAGGAGGGGAGCAGGCCGGTGGGTCTCCAGACGTCTCGAACTATTTTACACAGT 360
CAAAAGAGAAGCTTTGGTGAGAAGGCCCGAAATTTTTTGAAGGAGCACGAATATCTCGAG 420
GTATTATCATTACCAAGGATACGATTTAGGAAGGCTTGTGCTTCTTCTAGCATTATAGGT 480
GCTAGAAGTGGAGAGAAAGAGAAAGAGAAAGAGAAAAGGAGTAATGTCCAAAAGCTATT 540
ACGAGCTCCCAAGACTTGGATGCAAATTGTATTTCTAAACACGTAAATCGAAAAACGAAA 600
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 643
```

[5'3' Frame 1](#)

```
RK MetLRVGCLARSIVLTRGVTRRHFSSAPPEQQWEHILDR
LVCPLDKAALRAHRDDSGKLIELVNDRIGVAYPIIRGVPHL
TPADARALNPAAQQS MetKAAQKGEEGSRPVGLQTSRTIL
HSQKRSFG EKARNFLKEHEYLEVLSLPRI RFRKACASSSII
GARS GEKEKEKEKRR StopCPKAITSSQDL DANCISKHVNR
KTK
```

**SignalP-NN result:**# [data](#)

```
>Sequence          length = 70
# Measure  Position  Value  Cutoff  signal peptide?
max. C      19       0.302  0.32   NO
max. Y      19       0.346  0.33   YES
max. S       7       0.822  0.87   NO
mean S     1-18      0.520  0.48   YES
           D      1-18      0.433  0.43   YES
# Most likely cleavage site between pos. 18 and 19: TRG-VT
```

**SignalP-HMM result:**# [data](#)

&gt;Sequence

Prediction: Non-secretory protein

Signal peptide probability: 0.352

Signal anchor probability: 0.000

Max cleavage site probability: 0.181 between pos. 18 and 19

**Query=** local sequence:

(178 letters)

[5'3' Frame 2](#)AKCCALGALQGVLFSHVALLEGTLALHPQNSNGSTY **Stop** I

DLFAHLIKLPCARTVTTRENS **Stop** SLSTTG **Stop** GLHTR **Stop** Y  
VAFPIHQPTRVPSTRQPSRA **Stop** RPLRRERRGAGRWSR  
RLELFYTVKREALVRRPEIF **Stop** RSTNISRYHYQGYDLGR  
LVLLAL **Stop** VLEVERKRRKRKRG GNVQKLLRAPKTW **Met** Q  
IVFLNT **Stop** IEKR

### 5'3' Frame 3

QNAARWVPCKEYCSHTWRY **Stop** KAL **Stop** LCTPRTA **Met** GA  
HIR **Stop** TCLPT **Stop** **Stop** SCPARAP **Stop** RLGKTHRACQRPDR  
GCIPDNTWRSPSYTSRRACPQPGSPAHEGRSEGRGGEQ  
AGGSPDVSNYFTQSKEKLW **Stop** EGPKFFEGARISRGIIITK  
DTI **Stop** EGLCFF **Stop** HYRC **Stop** KWRE REREKEV **Met** SKSY  
YELPRLGCKLYF **Stop** TRKSKNE

### 3'5' Frame 1

FRFSIYVFRNTICIQVLGARN SFWTLPPFLFLFLSTSSY  
NARRSTSLPKSYPW **Stop** **Stop** **Stop** YLEIFVLLQKISGLLTkas  
LLTV **Stop** NSSRRLETHRPAPLLSLLSGLHALLGCRVEGTRV  
GWCK **Met** GNATYYRVCNPYPVVDKLYEFSRVVTVRAQGSF  
IKWANKSI **Stop** YVLPLLFWGCRAKVPSSNATCENNTPCKA  
PNAQHFA

### 3'5' Frame 2

FVFRFTCLEIQFASKSWELVIAFGHYLLFSFSFSFSPLLAPI  
**Met** LEEAQAFILNRILGNDNTSRYSCSFKKFRAFSPKLLF **Stop**  
LCKIVRDVWRPTGLLPSSPF **Stop** AAF **Met** LCWAAGLRARAS  
AGVRWGTPRIIGYATPIRSLTSS **Met** SFPESSRCARRAALS  
SGQTSLSN **Met** CSHCCSGGAELKCLLVTPRVRTILLARHPT  
RSIL

### 3'5' Frame 3

SFFDLRV **Stop** KYNLHPSLGSS **Stop** **Stop** LLDITSFSLSLSLSL



HF **Stop** HL **Stop** C **Stop** KKHKPS **Stop** IVSLV **Met** IIPRDIRAPSKNF  
G PSHQSFSFDCVK **Stop** FETSGDPPACSPPLPSERPSCSAG  
LPG **Stop** GHARRLV **Stop** DGERHVLSG **Met** QPLSGR **Stop** QAL  
**Stop** VFPSRHGARAGQLYQVGKQVYLICAPIAVLGVQS **Stop** S  
AF **Stop** **Stop** RHV **Stop** EQYSLQGTQRAAFC

## NKA11

CAGCTCCGGCCGCCATGGCCGCGGGATACAGTAACATCGATGAGCCTGACGAGAGGGGG 60

GTACGATGCGTATATGACAGGCGTGGTTTTTGCTAAGCAGAGAGCGGTGGCCACGGAAG 120

F1

AACAACTCAAAACGATCGAAACTGCGTCTTTTTGATGGGTTGCGAACAGTCACCCATGA 180

CTATTACAAGGGACGAAGAAGCAGTAGATTTATCAAAAGTGTTTTTGGTTACCAATTTTC 240

R1

CCTCCGCATTACAAATGACACATTTCAAGAGGAAGGACGTTTCGGACCTAACATCCGAG 300

TGCGGTGGGTCTCGGACACGAGTTGCTTGGTCATTCTACAAACGCCGCAAAGGAAGGTG 360

ATGACGTGGCATCGACATGGATACTCGAGGTGGATCCGACCAGTACTGTGGATATGCT 418

## 5'3' Frame 1

VRCVYDRRGFC **Stop** AESGGHGRRTTQNDRKLRLFDGLRTV  
THDYYKGRRSSRFIKSVFGYQFSLRIHK **Stop** HISRGRTFRT  
**Stop** HPSAVGLGHELLGHSTNAAKGR **Stop** **Stop** RGI

## 5'3' Frame 2

YDAY **Met** TGVVFAKQRAVATEEQ LKTIENCVFL **Met** GCEQSP  
**Met** TITRDEEAVDLSKVFLVTNFP SAFTNDTFQEEGRFGPNI  
RVRWVSDTSCLVILQTPQKEGDDVAS

5'3' Frame 3

T **Met** R I **Stop** Q A W F L L S R E R W P R K N N S K R S K T A S F **Stop** W V A N  
 S H P **Stop** L L Q G T K K Q **Stop** I Y Q K C F W L P I F P P H S Q **Met** T H F K R K  
 D V S D L T S E C G G S R T R V A W S F Y K R R K R K V **Met** T W H

3'5' Frame 1

R C H V I T F L L R R L **Stop** N D Q A T R V R D P P H S D V R S E T S F L L K C V  
 I C E C G G K I G N Q K H F **Stop Stop** I Y C F F V P C N S H G **Stop** L F A T H Q  
 K D A V F D R F E L F F R G H R S L L S K N H A C H I R I V

3'5' Frame 2

D A T S S P S F C G V C R **Met** T K Q L V S E T H R T R **Met** L G P K R P S S **Stop**  
 N V S F V N A E G K L V T K N T F D K S T A S S S L V I V **Met** G D C S Q P I K K  
 T Q F S I V L S C S S V A T A L C L A K T T P V I Y A S Y

3'5' Frame 3

**Met** P R H H L P F A A F V E **Stop** P S N S C P R P T A L G C **Stop** V R N V L P L  
 E **Met** C H L **Stop Met** R R E N W **Stop** P K T L L I N L L L L R P L **Stop Stop** S W  
 V T V R N P S K R R S F R S F **Stop** V V L P W P P L S A **Stop** Q K P R L S Y T H  
 R

**NKA 12**

CAGCTCCGGCCGCCATGGCCGCGGAT**TACAGTAACATCGATGAGCCTGACGAGAGGGGGG** 60  
 AGAAGATCACTGCTTTTGAAGACTATGTACAATCTATGGACGTTGCTGCCTTCAACAAGA 120  
 TCTAAAAAAGACAATAAATCATATAAACAGCAAAAAAAAAAAGCTCG**ACATGGATGC** 180  
**TCAAGGTGGATCCGACCAGTACTGTGGATATG** 212

**Match in Genbank Data Base:**

>[gi|14043203|gb|BC007590.1](#) **LU** Homo sapiens ribosomal protein, large,  
 P1, mRNA (cDNA clone  
 MGC:15616 IMAGE:3343021), complete cds  
 Length = 504

Score = 56.0 bits (28), Expect = 2e-05

Identities = 40/44 (90%)  
Strand = Plus / Plus

Query: 27 gaggagtctgatgacgatatgggctttggactttttgattaaac 70  
          |||||  
Sbjct: 404 gaggagtctgatgatgacatgggctttggtcttttggactaaac 447

### 5'3' Frame 1

RRSLLSKT **Met** YNLWTLLPSTRSKKRQ **Stop** **II** **Stop** TAKKKKA

### 5'3' Frame 2

EDHCFRRLCTIYGRCCLQQDLKKDNKSYKQQKKKKL

### 5'3' Frame 3

KITAFEDYVQS **Met** DVAAFNKI **Stop** KKTINHINSKKKKSS

### 3'5' Frame 1

RAFFFFAVY **Met** IYCLFLDLVEGSNVHRLYIVFESSDL

### 3'5' Frame 2

ELFFFLLFI **Stop** FIVFF **Stop** ILLKAATSIDCT **Stop** SSKAVIF

### 3'5' Frame 3

SFFFFCCLYDLLSFFRSC **Stop** RQQRP **Stop** IVHSLRKQ **Stop** S  
S

## NKA13

GCGACGCAGCTCCGGCCGCCATGGCCGCGGGAT **TACAGTAACATCGATGAGCCTGACGAG** 60  
**AGGGGGG**AACTTAATACCTTTTATTCCCCTTCATTAGCGCCTATCTTCAACAGCTTCTGC 120  
AGTAATTAAACCACACAATGCCACCACCTGCTTTGTGATGTAAATAAAGCATACTTACCA 180  
AAAAAAAAAAAAAAAAAGCTCG **ACATGGATACTCGAGGTGGATCCGACCAGTAC** 232

[5'3' Frame 1](#)

NLIPFIPLH **Stop** RLSSTASAVIKPHNATTCFV **Met Stop** IKHTY  
QKKKKKA

[5'3' Frame 2](#)

T **Stop** YLLFPFISAYLQQLLQ **Stop** LNHT **Met** PPPAL **Stop** CK **Stop**  
SILTKKKKKKL

[5'3' Frame 3](#)

LNTFYSPSLAPIFNSFC SN **Stop** TTQCHLLCDV NKAYLPKK  
KKKSS

[3'5' Frame 1](#)

RAFFFFFW **Stop** VCFIYITKQVVALCGLITAEAVEDRR **Stop**  
**Stop** RGIKGIK

[3'5' Frame 2](#)

ELFFFFFFGKYALFTSQSRWWHCVV **Stop** LLQKLLKIGANEG  
E **Stop** KVLS

[3'5' Frame 3](#)

SFFFFFFLV S **Met** LYLHHKAGGGIVWFNYCRSC **Stop** R **Stop** AL  
**Met** KGNKRY **Stop** V

**NKA 14**

CAGCTCCGGCCGCCATGGCCGCGGGAT **T**ACAGTAACATCGATGAGCCTGACGAGAGGGGA 60

F1

R1

GAGGAAGGTCGTGGAGGAGAAGAAGGAGGAGTCTGATGACGATATGGGCTTTGGACTTTT 120

TGATTAACTATGTTCCGGAAATAAATGTAGTCTTAAGCATAAAAAAAAAAAAAAAAAAAAAA 180

AAAAAAAAAAAAAAAAAAAAAGCTCG **ACATGGATACTCGAGGTGGATCCGACCAGTAC** 238

[5'3' Frame 1](#)

RGRSWRRRRRSL **Met** TIWALDFLIKLC SGNKCSLKHKKKKK  
KKKKKKKKSS

[5'3' Frame 2](#)

E EGRGGE EGV **Stop Stop** RYGLWTF **Stop** LNYVPEINVVLSIK  
KKKKKKKKKKKA

[5'3' Frame 3](#)

RKVVEEKKEESDDD **Met** GFGLFD **Stop T Met FRK Stop Met Stop S**  
**Stop A Stop** KKKKKKKKKKKKKL

[3'5' Frame 1](#)

RAFFFFFFFFFFFFFF **Met** LKTTFISGT **Stop** FNQKVQSPYRHQ  
TPPSSPPRPSS

[3'5' Frame 2](#)

ELFFFFFFFFFFFFFFFLCLRLHLFPEHSLIKKSKAHIVIRLLLLL  
LHDLP

[3'5' Frame 3](#)

SFFFFFFFFFFFFFFFYA **Stop** DYIYFRNIV **Stop** SKSPKPISSD  
SSFSSSTTFL

**NKA 15**

GCGATCCAGCTCCGCCGCCATGGCCGCGGGAT**TACAGTAACATCGATGAGCCTGACGAG** 60

F2

**AGGGGGG**ATGAAGCGAAATGTACACTACATTCAACGACGTGCCGTGGACGACAAATTCAT 120

R2

CAAGTAATTCGAATAGGCATTCCACCCTGTAATATCTTGCTGTAATATTTCTAATACATA 180

ATTTAAAATTAAA 240

284

**Met**KRNVHYIQRRVDDKFIK**Stop**FE**Stop**A FHPVISCCNISNT**Stop**FKIKKKKKKKKKKKKKKKKKKK

**Stop** SE **Met** Y T T F N D V P W T T N S S S N S N R H S T L **Stop** Y L A V I F L I  
H N L K L K K K K K K K K K K K K K K K K

EAKCTLHSTTCRGRQIHQVIRIGIPPCNILL **Stop** YF **Stop** YII  
**Stop** N **Stop** KKKKKKKKKKKKKKKKKKK

FFFFFFFFFFFFFFFFFFFF **Stop** F **Stop** | **Met** Y **Stop** KYYSKILQGG  
**Met** PIRIT **Stop** **Stop** | CRPRHVVECSVHFAS

FFFFFFFFFFFFFFFFNFKLCIRNITARYYRVECLFELLD  
EFVVHGTSLNVVYISLH

FFFFFFFFFFFFFFFFFLILNYVLEILQQDITGWNAYSNYL  
**Met** NLS STARR **Stop Met Stop** CTFRF

ACGAGCTCATGGATGACGTGGCACCACCAAACAGGAAAACCCAAATCACAACCGACAC 120

CAAATAAAACGCAACCGACGACGAGTAAAGCAAATGGCAAATCAACACCTGCCGCAAACG 180

R1

GAAACACTAAAAATGCGAAAGGAGGTGCTCCACAGAAAAAACTGCGACTAAGCGGCCGCT 240

AAGCTTAGCGACCAGTAC

258

### [5'3' Frame 1](#)

DEL **Met** DDVAPPKQEKP KSQPTPNKTQPTTSKANGKSTPA  
ANGNTKNAKGGAPQKKL

### [5'3' Frame 2](#)

TSSW **Met** TWHHQN RKNPNHNRHQIKRNRRRVKQ **Met** ANQH  
LPQTETLK **Met** RKEVLHRKNC

### [5'3' Frame 3](#)

RAHG **Stop** RGTTKTGKTQITTDTK **Stop** NATDDE **Stop** SKWQIN  
TCRKRKH **Stop** KCERRCSTEKTA

### [3'5' Frame 1](#)

RSFFCGAPPFAFLVFPFAAGVDLPFALLVVGCVLFGVGC D  
LGFSCFGGATSS **Met** SS

### [3'5' Frame 2](#)

AVFSVEHLLSHF **Stop** CFRLRQVLICHLLYSSSVAFYLVSVVI  
WVFPVLVVP RHP **Stop** AR

### [3'5' Frame 3](#)

QFFLWSTSFRIFSVSVCGRC **Stop** FAICFTRRRLRFIWCRL  
**Stop** FGFFLFWWCHVIHEL V

**NKA 19**

TGCAATCCAGCTCCGGCCGCCATGGCCGCGGGAT<sup>T</sup>ACAGTAACATCGATGAGCCTGACGA 60  
GAGGGGGGAGGTGGGGTAACATGCCGGAGGGGCGAGCATCAAGGCTAGGCTCCACCACCA 120  
TGGTCGTCCCAAAAAAAAAAAAAAAAAAGCTCGACATGGATACTCGAGGTGGATCCGACCAG 180  
TACTGTGGATATGCT

**5'3' Frame 1**

RWGN**Met**PEGRASRLGSTT**Met**VVPKKKKKA

**5'3' Frame 2**

GGVTCRRGEHQG**Stop**APPPWSSQKKKKKL

**5'3' Frame 3**

VG**Stop**HAGGASIKARLHHHGRPKKKKKSS

**3'5' Frame 1**

RAFFFFFFGTT**Met**VVEPSLDARPSG**Met**LPH

**3'5' Frame 2**

ELFFFFLGRPWWWSLAL**Met**LAPPACYPT

**3'5' Frame 3**

SFFFFFFWDDHGGGA**Stop**P**Stop**CSPLRHVTPP

**NKA 20**

CAGCTCCGGCCGCATGGCCGCGGGAT<sup>T</sup>CAGTAACATCGATGAGCCTGACGAAGAACAACC 60  
F1  
AGACTACGCCCGGGTCACTTTCGAGAGTGGCGCAGTAGAGAACTATGACATAGTTATCGG 120  
R1  
TGCTGATGGACTCGGATCTCGACATGGATACTCGAGGTGGATCCGACCAGTAC 173



[5'3' Frame 1](#)

RTTRLRPGHFREWRSREL **Stop** HSYRC **Stop** WTRIS

[5'3' Frame 2](#)

EQPDYARVTFESGAVENYDIVIGADGLGS

[5'3' Frame 3](#)

NNQTTPGSLSRVAQ **Stop** RT **Met** T **Stop** LSVL **Met** DSDL

[3'5' Frame 1](#)

RDPSPSAPIT **Met** S **Stop** FSTAPLSKVTRA **Stop** SGCS

[3'5' Frame 2](#)

EIRVHQHR **Stop** LCHSSLLRHSRK **Stop** PGRSLVV

[3'5' Frame 3](#)

RSESISTDNYVIVLYCATLESDPGVVWLF

**NKA 22**

GGCCAATCAGCTCCGGCCGCCATGGCCGCGGGAT<sup>T</sup>ACAGTAACATCGATGAGCCTGACGA 60  
GAGGGGGCAGAAATGAAGAAGGAGCGAGAAGAGAAGGAGAGGAAGGCACGAGAGGAAGCC 120  
AAGAAAAACCAAAAAAAAAAAAAAAAAAAGCTCG<sup>A</sup>CTAAGCGCCGCTAAGCTTAGCGAC 179

[5'3' Frame 1](#)

RNEEGARREGEEGTRGSQEKPKKKKKKL

[5'3' Frame 2](#)

E **Met** KKEREKERKAREEAKKNQKKKKKSS

[5'3' Frame 3](#)

K **Stop** RRSEKRRRGRHERKPRKTKKKKKKA

[3'5' Frame 1](#)

RAFFFFFLVFLGFLSCLPLLLFSLLLHF

[3'5' Frame 2](#)

ELFFFFFFWFFLASSRAFLSFSSRSFFIS

[3'5' Frame 3](#)

SFFFFFFFGFSWLPLVPSSPSLLAPSSF

**NKA23**

CAGCTCCGGCCGCATGGCCGCGGGATTCAGTAACATCGATGAGCCTGACGAGAGGGGGA 60

GATGAGGATGAGGAGGAGGAGGAAGAGGAGGAGGAACATTTTACACAAATAAATTCTC 120

TGTAACCCCAAAAAAAAAAAAAAAAAAGCTCGACATGGATACTCGAGGTGGATCCGACCAGT 180

AC 182

[5'3' Frame 1](#)

R **Stop** G **Stop** G G G R G G G T H F T Q I N S L **Stop** P Q K K K K K L

[5'3' Frame 2](#)

D E D E E E E E E E E L I L H K **Stop** I L C N P K K K K K S S

[5'3' Frame 3](#)

**Met** R **Met** R R R R K R R R N S F Y T N K F S V T P K K K K K A

[3'5' Frame 1](#)

RAFFFFFFGV TEN L F V **Stop** N E F L L L F L L L L I L I

[3'5' Frame 2](#)

ELFFFFLGLQRIYLCK **Met** SSSSSSSSSSSSSSS

[3'5' Frame 3](#)

SFFFFFFWGYREFICVK **Stop** VPPPLPPPPHPH

**NKA27**

CAGCTCCGGCCGCCATGGCCGCGGGAATACAGTAACATCGATGAGCCTGACGAGAGGGGG 60

GACCAGACAAAGTGGGCAAGTCTACGCTCAACATGGCGACGCTTCAAGAACGAAATCCA 120

AAAAAAAAAAAAAAAAAGCTCGACTAAGCGGCCGCTAAGCTAGCGACCAGTAC 172

[5'3' Frame 1](#)

TRQSGQVYAQHGD AFKNEIQKKKKKA

[5'3' Frame 2](#)

PDKVGKSTLN **Met** ATLSRTKSKKKKKKL

[5'3' Frame 3](#)

QTKWASLRSTWRRFQERNPKKKKKSS

[3'5' Frame 1](#)

RAFFFFFFWISFLKASPC **Stop A Stop** TCPLCL

[3'5' Frame 2](#)

ELFFFFFFGFRS **Stop** KRRHVERRLAHFVW

[3'5' Frame 3](#)

SFFFFFFLDFVLESVA **Met** LSVDLPTLSG

**NKA 35**

ACCCATCCAGCTCCGGCCGCCATGGCCGCGGGAT<sup>T</sup>ACAGTAACATCGATGAGCCTGACGA 60  
GAGGGGGG GATAAAACAAGGATTAAAAGTACATTTTGTGGATTATTATCATGATATTTTT 120  
AAGGTGGCTTTTCCCAATCTGAAGCCAATGGAAAGAAAATTAAATTGACCGGTTGTACAA 180  
TAAAAAAAAAAAAAAAAAAAAAGCTCGACATGGATACTCGAGGTGGATCCGACCAGTAC 240

**5'3' Frame 1**

DKTRIKSTFCGLLS **Stop** YF **Stop** GGFSQSEANGKKIKLTGCTI  
KKKKKKKA

**5'3' Frame 2**

IKQGLKVHFVDYYHDIFKVAFPNLKP **Met** ERKLN **Stop** PVVQ  
**Stop** KKKKKKKL

**5'3' Frame 3**

**Stop** NKD **Stop** KYILWIII **Met** IFLRWLFPI **Stop** SQWKEN **Stop** IDR  
LYNKKKKKKKSS

**3'5' Frame 1**

RAFFFFFFFIVQPVNLIFFPLASDWEKPP **Stop** KYHDNNPQN  
VLLILVL

**3'5' Frame 2**

ELFFFFFFFLYNRSI **Stop** FSFHWLQIGKSHLKNI **Met** IIIHK **Met**  
YF **Stop** SLFY

**3'5' Frame 3**

SFFFFFFFYCTTGQFNFLSIGFRLGKATLKIS **Stop Stop Stop** S  
TKCTFNPCFI

**NKA 37 (= NKA38)**

CTGCTCCGGCCGCCATGGCCGCGGGAT**TACAGTAACATCGATGAGCCTGACGAGAGGGGG** 60

**G**AGGAACTCAGGAGACAGGTGGGAGACGCTAAGAAAAAAGACAAGGAAAGGGGACAAGAG 120

CTGGAGGCGGAAATGAAGAAAAAAGAGCG**ACTAAGCGGCCGCTAAGCTTAGCCGACCAGT** 180

**ACTGTGGATATGCTTAC**

**5'3' Frame 1**

RNSGDRWETLRKKTRKGDKSWRRK **Stop** RKKS

**5'3' Frame 2**

GTQETGGRR **Stop** EKRQKGKTRAGGGNEEKRA

**5'3' Frame 3**

ELRRQVGDAKKKDKERGQELEAE **Met** KKKE

**3'5' Frame 1**

RSFFFISASSSCPLSLSFFLASPTCLLSS

**3'5' Frame 2**

ALFSSFPPPALVPFPCLFS **Stop** RLPPVS **Stop** VP

**3'5' Frame 3**

LFFLHFRLQLLSPFLVFFLSVSHLSPEF

**NKA43**

CGCTCCGGCCGCCTGGCCGCGGGAT**TACAGTAACATCGATGAGCCTGACGAGAGGGGGG**GA 60

AGGAGAGGAAGAGATGGAAGAAGCAGTACCAGAAGATGAAATACAAGAGGGAGAGGAGCA 120

F1

CACAGACGTATTACCAATAGAAATGCAGAACAAATGGGACACTGAACCACAAGCAGTTACC 180

GAACGAAGAACAACACGATATATGACAAAGTACGAACGTGCAAGAATTTTGGGAACCCGT 240  
GCACTTCAAATCAGCATGAATGCACCGATTATGGTCTGAACCTGGAAGGAGAGACAGACCCA 300  
CTGCAAATTGCCATGAAAGAGCTGCGTGAGCGCAAGATCCCTTTAATCATTTCGCAGATAT 360  
R1  
TTGCCGGATCAATCATATGAAGACTGGACTGTTGAGGAGCTTATCACCGATCAATTCTAA 420  
AACTATCTTTGTTCTAAAGCTTCTTTTGTAACTAATAAACTATAAACTATAAAAAA 480  
AAAAAAAAAAAAAGCTCGACATGGATACTCGAGGTGGATCCGACCAGTAC 530

**Match in Genebank Data Base:**

[gi|4960151|gb|AF153278.1|AF153278](#) Nicotiana tabacum DNA-directed RNA  
polymerase IIb (NT193) mRNA,  
complete cds  
Length = 830

Score = 58.0 bits (29), Expect = 1e-05  
Identities = 56/65 (86%)  
Strand = Plus / Plus

Query: 141 tatatgacaaagtacgaacgtgcaagaatTTTGGGAACCCGTgcacttcaaatcagcatg 200  
||||||| || ||||| |||| |||| || ||||| || |||||  
Sbjct: 263 tatatgacaaaatatgaacgtgctagaatcctgggcactcgtgcactccagatcagcatg 322

Query: 201 aatgc 205  
|||||  
Sbjct: 323 aatgc 327

**5'3' Frame 1**

EGEEE **Met** EEAVPEDEIQEGEEHTDVLPIE **Met** QNKWTLNHK  
QLPNEEQHDI **Stop** QSTNVQEFWEPVHFKSA **Stop Met** HRLWS  
NWKERQTHCKLP **Stop** KSCVSARSL **Stop** SFADICRINH **Met** KT  
GLLRSLSPINSKTIFVLKLLL **Stop H Stop Stop** TIKL **Stop** KKKKK  
KA

[5'3' Frame 2](#)

KERKRWKKQYQK **Met** KYKRERSTQTYYQ **Stop** KCRTNGH  
**Stop** TTSSYRTKNNTIYDKVRTCKNFGNPCTSNQHECTDYG  
RTGRRDRPTANCHERAA **Stop** AQDPFNHSQIFAGSII **Stop** RL  
DC **Stop** GAYHRSILKLSLF **Stop** SFFCNTNKL **Stop** NYKKKKKK  
KL

[5'3' Frame 3](#)

RRGRDGRSSTRR **Stop** NTRGRGAHRRITNRNAEQ **Met** DTEP  
QAVTERRTTRY **Met** TKYERARILGTRALQIS **Met** NAPI **Met** VEL  
EGETDPLQIA **Met** KELRERKIPLIIRRYLPDQSYEDWTVEEL  
ITDQF **Stop** NYLCSKASFVTLINYKTIKKKKKKSS

[3'5' Frame 1](#)

RAFFFFFFFYSFIVY **Stop** CYKRSFRTKIVLELIGDKLLNSPVF  
I **Stop** LIRQISAND **Stop** RDLALTQLFHGNLQWVCLSFQFDHN  
RCIHADLKCTGSQNSCTFVLCHISCCSSFGNCLWFSVHLF  
CISIGNTSVCSSPSCISSSGTASSISSP

[3'5' Frame 2](#)

ELFFFFFFFIVL **Stop** FISVTKEALEQR **Stop** F **Stop** N **Stop** SVISSS  
TVQSSYD **Stop** SGKYLR **Met** IKGILRSRSSF **Met** AICSGSVSPS  
SSTIIGAF **Met** LI **Stop** SARVPKILARSYFVIYRVVLRSVTACG  
SVSICSAFLLVIRLCAPLPLVFHLLVLLLPSLPLL

[3'5' Frame 3](#)

SFFFFFFFL **Stop** FYSLLVLQKKL **Stop** NKDSFRIDR **Stop** **Stop** AP  
QQSSLH **Met** IDPANICE **Stop** LKGSCAHAALSWQFAVGLSLL  
PVRP **Stop** SVHSC **Stop** FEVHGFPKFLHVRTL SYIVLFFVR  
**Stop** LLVVQCPFVLHFYW **Stop** YVCVLLSLLYFIFWYCFFHLF  
LSF

GGCAACAAGCTTGATTCAAAATGTCCGTCCAACAAGGAGTTTCTGGTGAGCCCGCAAGAG	60
F1	
GAGAAGTGTTTCTCTACAAAGATGCTA <u>ACTTCAGCGGAAACTCATGGAAAGTTACTGGAA</u>	120
ATGTATTTGATTTCCGTAGTGTCTCTGGTTTAAATGATGTGGTATCTTCCGTAAAGTGG	180
GACCTAACACTAAGGCCTTCATCTTCAAGGATGACCGATTCAATGGCGATTTTATTCGCC	240
R2	R1
<u>TGGAGCAGAACACTCAAGTGACTGATCTCACC</u> ACTCGCAATTTAAAC <u>GACGCCATTT</u> CAT	300
<u>CCATCATCGTGGCCACTTTTGATAGCGCTTAAAGCATACTACAATAAATAGTTGATATGA</u>	360
TTTTTTTTTAGTAAAAAAAAAAAAAAAAAAAAAAAAAAGCTCG	403

Length = 391

NKA48: 34 aaggagtttctggtagcccgcaagaggagaagtgtttctctacaaagatgctaacttca 93  
 |||||  
 Sbjct: 57 aaggagtttctggaaaccagcaaaaggggaggtgtttctgtacaagcacgttaacttcc 116

NKA48: 94 gcggaaactcatggaaagtactggaaatgtatttgatttccgtagtgctctggtttaa 153  
 |||  
 Sbjct: 117 agggagattcatggaaagtactggcaatgtctatgatttccgtagtgctctggtttaa 176

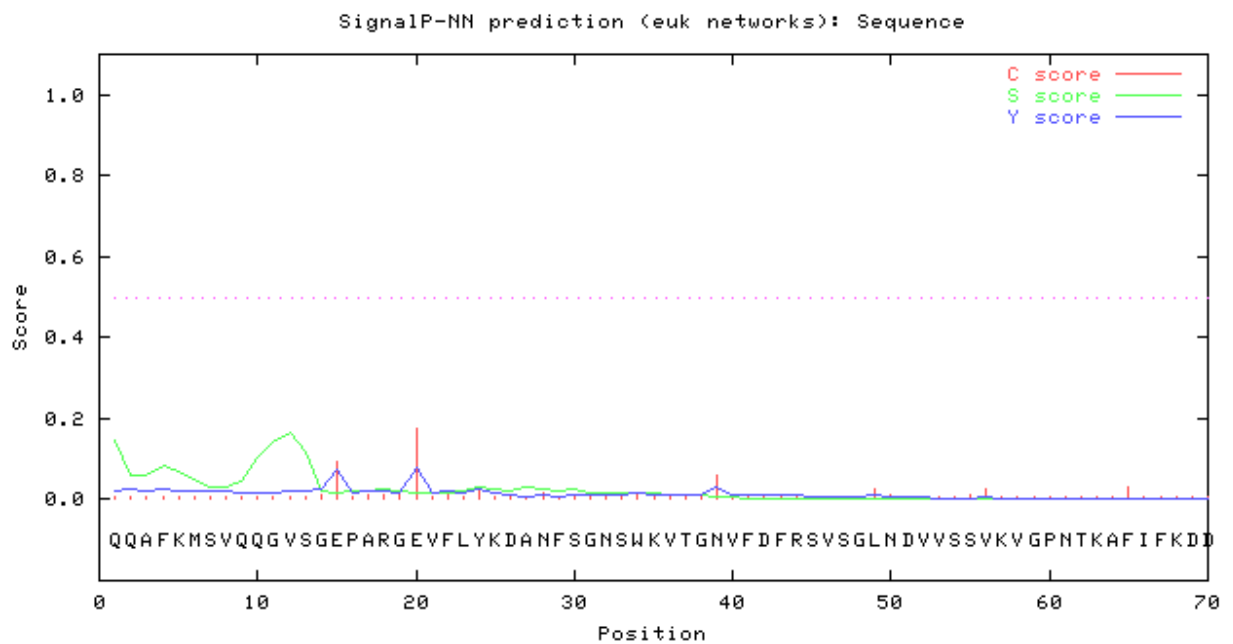
NKA48: 154 atgatgtggtatcttccgttaaagtgggacctaacactaaggccttcatcttcaaggatg 213  
 |||||  
 Sbjct: 177 atgatgttggtcctctgttaaagtggggcctaacactaaggccttcatcttcaaggacg 236

NKA48: 214 accgattcaatggcgtattttattcgctggagcagaacactcaagtgactgatctcacca 273  
 ||  
 Sbjct: 237 acaggttcaacggcaattttattcgcttggaggagagctcgcaagtgactgatctcacca 296

NKA48: 274 ctcgcaatttaaacgacgccatttcatccatcatcgctggccacttttgatagcgc 328  
 |||||  
 Sbjct: 297 ctcgcaacttgaacgatgccatttcgtccatcatcgctggctacgtttgagagcgc 351



SignalP-NN result:



# [data](#)

>Sequence            length = 70

# Measure   Position   Value   Cutoff   signal peptide?

max. C    20      0.176   0.32   NO

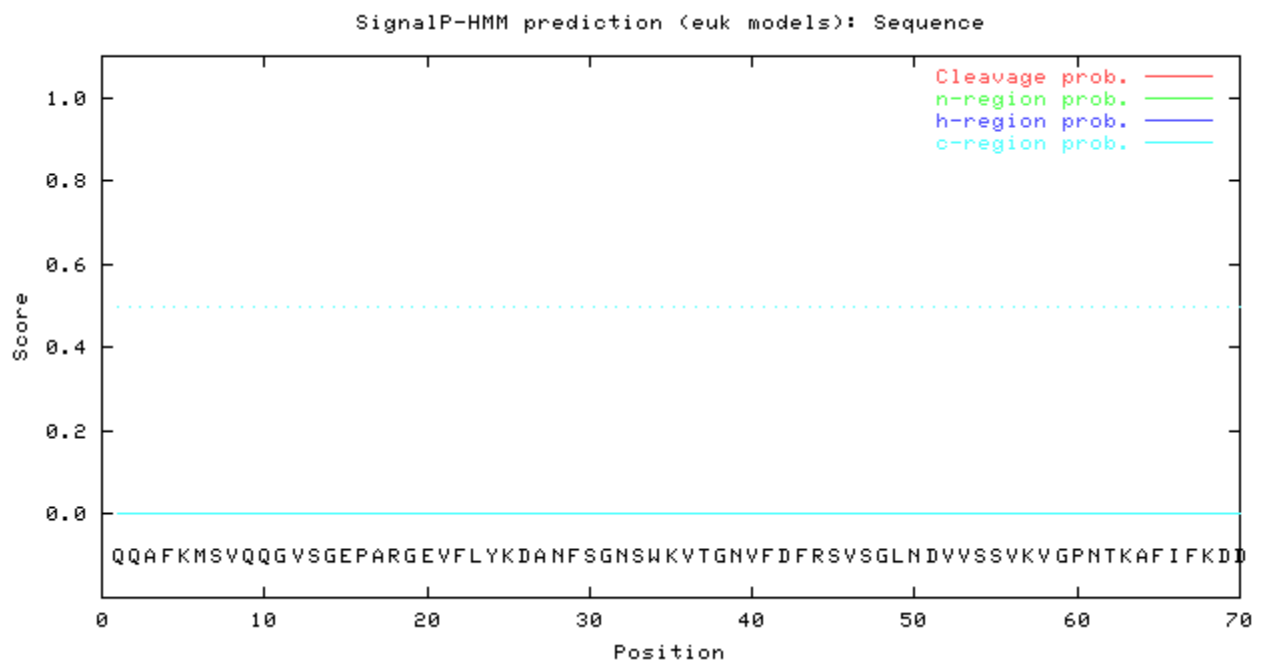
max. Y    20      0.080   0.33   NO

max. S    12      0.168   0.87   NO

mean S    1-19    0.065   0.48   NO

D    1-19    0.072   0.43   NO

SignalP-HMM result:



# [data](#)

>Sequence

Prediction: Non-secretory protein

Signal peptide probability: 0.002

Signal anchor probability: 0.000

Max cleavage site probability: 0.002 between pos. 19 and 20

**NKA49**

CAGCTCCGGCCGCCATGGCCGCGGGGAT<sup>T</sup>ACAGTAACATCGATGAGCCTGACGAGAGGGG 60

F1

GGGGAGGGGAAATCGTAGGTCTGGAGCTGGCGCGAGATACTGTCTTCCACGACGGAAACG 120

R2

ATGACGAAATCGGTAGGGTTGACTTGATGAATGTCCGACTTGTACGCAATTCGATTGAG 180

R1

GTTCCACCTACGTCAGCATGTGTTGGAGAGCCAAAAAAAAAAAAAAAAAGCTCGACATGGAT 240

ACTCGAGGTGAATCCGACCAGTACCTGTGGATATGCT

**5'3' Frame 1**

GGEIVGLELARDTVFHDGNDDEIGRVDL **Met** NVRLVRNSD  
**Stop** GSTYVS **Met** CWRAKKKKKA

**5'3' Frame 2**

EGKS **Stop** VWSWREILSSTTET **Met** TKSVGLT **Stop Stop Met** SDL  
YAIPIEVPPTSACVGEPKKKKKL

**5'3' Frame 3**

RGNRRSGAGARYCLPRRK **Stop** RNR **Stop** G **Stop** LDECPTCT  
QFRLRFHLRQHVLESQKKKKSS

**3'5' Frame 1**

RAFFFFLALQH **Met** LT **Stop** VEPQSELRTSRTFIKSTLPISSSF  
PSWKTVSRASSRPTISP

**3'5' Frame 2**

ELFFFFWLSNTC **Stop** RRWNLNRNCVQVGHSSSQPYRFRH  
RFRRGRQYLAPAPDLRFPL

**3'5' Frame 3**

SFFFFFFGSPTHADVGGTSIGIAYKSDIHQVNPTDFVIVSVV  
EDSISRQLQTYDFPS

**NKA52**

CAGCTCCGGCCGCATGGCCGCGGGAT~~T~~ACAGTAACATCGATGAGCCTGACGAGAGGGGGG 60

GACGGCGAGGACAGAGACAGAAGGAGAGATGACAGAGACAAAATGAAAATAAACTCCATG 120

CCATCATAATAGTTACATAAAAAACCTTTACCCTTTCCCAAAAAAAAAAAAAAAAAAAGCTCG 180

ACATGGATACTCGAGGTGGATCCGACCAGTACTGTGGATATGCT 224

**5'3' Frame 1**

DGEDRDRRRDDRDK **Met** KINS **Met** PS **Stop Stop** LHKNLYPFPK  
KKKKSS

**5'3' Frame 2**

TARTETEGE **Met** TETK **Stop** K **Stop** TPCHHNSYIKTFTLSQKKK  
KKA

**5'3' Frame 3**

RRGQRQKER **Stop** QRQENKLNHAIIVT **Stop** KPLPFPKKKKK  
KL

**3'5' Frame 1**

RAFFFFFFWERVKVF **Met Stop** LL **Stop** WHGVYFHFVSVISPSVS  
VLAV

**3'5' Frame 2**

ELFFFFFFGKG **Stop** RFLCNYYDG **Met** EFIFILSLSSLLLSLSSP

**3'5' Frame 3**

SFFFFFFLGKGKGFYVTI **Met Met** AWSLFSFCLCHLSFCLCPR  
R

## Erklärung

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe.

Regensburg, den 26.10.2004

(Nadthanun Pinchai)